EFFECT OF EXERCISE AND CALORIC RESTRICTION ON RENAL DOPAMINE D1 RECEPTOR FUNCTION IN OBESE ZUCKER RATS

A Dissertation Presented to

Department of Pharmacological and Pharmaceutical Sciences

College of Pharmacy

University of Houston

In Partial Fulfillment of the

Requirement for the Degree

Doctor of Philosophy

In Pharmacology

By

Abdul Bari Muhammad

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Abstract

Obesity is a major risk factor for the development of hypertension and it is associated with hyperglycemia, hyperinsulinemia (type II diabetes), sodium retention and oxidative stress. However, the mechanisms involved in obesity related development of hypertension are not clearly understood. Development of hypertension can be explained in part by sodium retention resulting from impaired regulation of sodium homeostasis. Renal dopamine, acting via the renal dopamine D1 receptor (D1R), promotes the excretion of sodium and plays a vital role in the maintenance of sodium homeostasis.

Impairment of D1R function is associated with hypertension in humans and animal models including $Lepr^{fa/fa}$ Zucker **(**obese) rats. We have previously reported that treatment of these rats with antioxidants or insulin sensitizers reduced insulin levels and oxidative stress, restored D1R function and reduced blood pressure. Further, the redox sensitive transcription factor, nuclear factor κ B (NFκB) has been implicated in impairment of D1R function during oxidative stress.

We investigated the effect of exercise on insulin levels, oxidative stress, nuclear translocation of NFκB, blood pressure, albuminuria, and D1R function. The exercise protocol involved treadmill exercise from three weeks of age for eight weeks. Exercise reduced oxidative stress, nuclear translocation of NFκB and

albuminuria. However, exercise did not reduce plasma insulin levels or blood pressure. Also, selective D1R agonist (SKF38393) mediated increases in GTPγS binding and sodium excretion were impaired in obese rats compared to lean rats and exercise did not restore this defect. We concluded that, although exercise is beneficial in reducing oxidative stress and renal injury, reducing insulin levels may be required to restore D1R function in obese rats. This hypothesis is supported by our findings that three-week-old $Lepr^{fa/fa}$ Zucker **(**obese) rats do not display oxidative stress, increased nuclear translocation of NFκB, or elevated blood pressure. However, they display hyperinsulinemia and impaired inhibition of Na^+/K^+ ATPase in response to D1R agonist.

Caloric restriction has been shown to attenuate hyperinsulinemia in obese rats. We hypothesized that caloric restriction in obese rats, starting from an early age, will reduce insulin levels, restore D1R function, and reduce blood pressure. Caloric restriction in obese rats reduced insulin levels by ~50%. However, it did not reduce oxidative stress, did not restore D1R function, or reduce blood pressure.

Therefore, we conclude that both hyperinsulinemia and oxidative stress can independently impair renal dopamine D1 receptor function and contribute to the development of hypertension in obese rats. Simultaneously reducing both

oxidative stress and hyperinsulinemia might be required to restore renal dopamine D1 receptor function and lower blood pressure.

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LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
AChE	Acetylcholine Esterase
ANP	Atrial natriuretic peptide
BHT	Butylated hydroxy-toluene
BMI	Body mass index
BSA	Bovine serum albumin
BSO	Buthionine sulfoxime
CER	Cytoplasmic extraction reagent
$CL_{creatinine}$	Creatinine clearance
COX	Cyclo-oxygenase
D1R	Dopamine D1 receptor
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DNP-hydrazone	2,4-dinitrophenylhydrazone
DNPH	2,4-dinitrophenylhydrazine
DTNB	5,5'-Dithiobis-2-nitrobenzoic acid

DS	Dahl salt sensitive rats
EDTA	Ethylene-diamine-tetra-acetic acid
EIA	Enzyme immunosorbent assay
ELISA	Enzyme linked immunosorbent assay
FE_{Na}	Fractional excretion of sodium
GFR	Glomerular filtration rate
GPCR	G protein coupled receptors
GRK	G protein-coupled receptor kinase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GTPγS	Guanosine 5'-O-[gamma-thio]triphosphate
H_2O_2	Hydrogen peroxide
HO-1	Hemeoxygenase-1
IP3	Inositol-tris-phosphate
KHB	Krebs–Henseleit buffer
L-DOPA	L-3,4-dihydroxyphenylalanine
MDA	Malondialdehyde

MAP	Mean arterial pressure
NADPH	Nicotinamide adenine dinucleotide phosphate
NaPi2	Na^+/phosphate co-transporter type 2
NER	Nuclear extraction reagent
NHE3	Na^+/H^+ exchanger type 3
NFκB	Nuclear factor κ B
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline-tween
PEG	Polyethylene glycol
PKA	Protein kinase A
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PMSF	Phenyl methyl sulfonyl fluoride
PVDF	Polyvinylidene fluoride
QUICKI	Quantitative insulin sensitivity check index
RIA	Radio-immuno assay

ROS	Reactive oxygen species
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
SD	Sprague Dawley rats
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TBS	Tris-buffered-saline
TBS-T	Tris-buffered-saline with tween-20
TEMPOL	4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl
$U_{Na}V$	Urinary sodium excretion

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1. Introduction and statement of the problem:

Obesity is a major risk factor for the development of hypertension and it is associated with hyperglycemia, hyperinsulinemia (type II diabetes), sodium retention and oxidative stress (12, 47, 109, 140, 158, 185, 188, 190). However, the mechanisms involved in obesity related development of hypertension and accompanying complications are not clearly understood (60, 190, 191). Development of hypertension can be explained in part by impaired regulation of sodium homeostasis (37, 60, 103, 190). Numerous studies, both in animal models and humans, point towards an involvement of defective renal dopamine D1 receptor function in impairment of sodium homeostasis and pathogenesis of hypertension (121). In animal models, dopamine mediated natriuresis is diminished in obese Zucker rats (111, 137, 143), spontaneously hypertensive rats (SHR) (50, 72) and Dahl salt-sensitive rats (DS) (100). This phenomenon is associated with decreased ability of dopamine to inhibit both Na^+/H^+ exchanger and Na^+/K^+ ATPase (110, 111, 137).

An increasing number of studies, in several models of hypertension as well as in old Fischer344 rats (64) and Sprague Dawley (SD) rats treated with pro-oxidants (30), indicate a critical role of oxidative stress in the impairment of dopamine D1 receptor function. In these models, natriuresis and inhibition of Na^+/K^+ ATPase in response to dopamine D1 receptor agonists is reduced (30, 64). This has been

attributed to dopamine D1 receptor uncoupling from G proteins and receptor downregulation (30, 64). Studies in both animal models and cell cultures have shown the involvement of nuclear factor κ B (NFκB), a redox sensitive transcription factor, in D1 receptor-G protein uncoupling (27, 63). Also, in these studies, antioxidant treatment prevented the nuclear translocation of NFκB leading to restoration of D1 receptor-G protein coupling, D1 receptor mediated inhibition of Na^+/K^+ ATPase and D1 receptor mediated natriuresis (27, 63, 64).

Adult obese Zucker rats that are used as a genetic model to study obesity-associated hypertension exhibit hyperglycemia, hyperinsulinemia and oxidative stress (4). Treatment of adult obese Zucker rats with both insulin sensitizers (rosiglitazone) and the antioxidant tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) decreased oxidative stress, improved insulin sensitivity, restored dopamine D1 receptor mediated natriuresis and reduced blood pressure (21, 32, 208-210).

Like antioxidants, exercise reduces oxidative stress in part by augmenting physiological anti-oxidant mechanisms (48, 126). Asghar et al (19) have shown that exercise augments antioxidant defenses, reduces oxidative stress and restores renal dopamine D1 receptor function in old Fischer344 rats. However, it is not known if exercise can reduce oxidative stress, prevent impairment of dopamine D1 receptor function and development of hypertension in obese

Zucker rats. Therefore, we wanted to investigate the influence of exercise on oxidative stress, insulin sensitivity, D1 receptor function and development of hypertension in obese Zucker rats.

Furthermore, reports indicate that overweight adolescents are about twenty times more likely than their leaner peers to be obese in adulthood (130). Therefore, preventing or reducing obesity early on in life may be more cost effective considering the enormous costs associated with management of comorbidities associated with obesity related hypertension (130). It is suggested that there are sensitive or critical periods during early life and interventions during this phase may be more likely to alter lifelong physiological processes than in adulthood (130). $Lepr^{fa/fa}$ (obese) Zucker rats at three weeks of age are of similar weight as their lean counterparts. Since obese Zucker rats express the obese phenotype only by 6-7 weeks of age, we hypothesized that exercising obese Zucker rats from three-four weeks of age will improve insulin sensitivity and prevent oxidative stress. These changes will prevent nuclear translocation of NFκB, impairment of D1 receptor mediated natriuresis and increase in blood pressure in adult obese Zucker rats.

Caloric restriction is another lifestyle intervention that is recommended for treatment of obesity related comorbidities (78, 130). Caloric restriction to reduce weight in hypertensive patients is associated with improvement of insulin

sensitivity and a reduction of blood pressure (155, 160). Recently, Metcalf et al (148) suggested that physical inactivity is a result rather than a cause for obesity and that interventions aimed at reducing weight should focus on reducing caloric intake. More importantly, in obese Zucker rats, hyperphagia is a major contributor to the acceleration of weight gain (16, 201). Caloric restriction in these rats is associated with decrease in weight gain (201, 225) and decrease in hyperinsulinemia (22, 141). Caloric restriction, in both humans and animal models, is associated with a decrease in oxidative stress (39, 120, 123, 200, 211, 224). However, in the presence of disease states, caloric restriction may not reduce oxidative stress as effectively or even be detrimental (173, 200). Literature addressing the ability of caloric restriction to reduce blood pressure in obese Zucker rats is limited and offers conflicting results (131, 141). It is not known if caloric restriction can prevent the impairment of dopamine D1 receptor function in obese Zucker rats.

Starting caloric restriction at an early age may offer greater benefit (130, 141). However, because of concerns that restricting calories at 3-4 weeks of age may interfere with development, we decided to restrict calories starting at 6-7 weeks of age. In addition, based on the data from exercise study and three-week-old $Lepr^{fa/fa}$ (obese) Zucker rats, we hypothesized that restricting caloric intake in obese Zucker rats from 6-7 weeks of age for ten weeks will decrease insulin

levels, restore dopamine D1 receptor mediated natriuresis, and reduce blood pressure.

2. REVIEW OF LITERATURE:

2.1 Role of Kidney in Sodium Homeostasis:

Kidney plays a vital role in the maintenance of sodium homeostasis, extracellular fluid volume and blood pressure (90, 91, 146, 222). The kidney is composed of functional units called the nephron. The kidney regulates sodium homeostasis and extracellular fluid volume by means of filtration and subsequent reabsorption through a variety of sodium and water transporters found along the entire length of the nephron. The type of transporters, their relative abundance and extent of sodium and fluid reabsorption vary across the various segments of the nephron (147). The proximal tubular segment is responsible for reabsorption of ~70% of the glomerular filtrate (both sodium and water) (147). Sodium is absorbed passively at the apical surface of proximal tubular epithelium that expresses various sodium transporters like Na^+/H^+ exchanger type 3 (NHE3), Na^+/glucose co-transporter, Na^+/amino acid co-transporter and the Na^+/phosphate co-transporter type 2 (NaPi2) (147, 184). The loop of Henle at the luminal side, passively reabsorbs approximately another 20% of the sodium primarily via the $Na^+/K^+/2Cl^-$ co-transporter (54, 87, 92, 184). In the distal tubule, sodium is reabsorbed passively at the luminal side primarily via the Na^+/Cl^- carrier (184). In the collecting tubules and collecting ducts, sodium is reabsorbed at the luminal side via NHE3 and sodium channels by a passive process (184). The

electrochemical gradient needed to maintain the above passive processes is primarily generated by the basolateral Na^+/K^+ ATPase (147, 184). Na^+/K^+ ATPase actively transports the sodium entering from the apical side, out of the epithelia and into circulation (184). Water reabsorption in all the segments, except the collecting tubules and ducts, occurs by paracellular diffusion and is coupled to electrolyte reabsorption (184). In the collecting tubules and ducts, presence of zona occludens (tight junctions) prevents paracellular diffusion (184). Here, water is passively reabsorbed transcellularly via aquaporins and is dissociated from electrolyte reabsorption (184).

The kidney is under the control of neuronal and hormonal factors (45, 146, 147, 223). Several hormones tightly regulate the various transporters and hence the extent of sodium and fluid reabsorption (15, 54, 92, 170, 216). Under normal conditions, anti-natriuretic hormonal factors like angiotensin II and norepinephrine predominate and promote reabsorption and maintain sodium homeostasis. However, under conditions of increased sodium intake anti-natriuretic hormones like dopamine and atrial natriuretic peptide (ANP) predominate and promote excretion of sodium (13).

2.2 Role of Dopamine in Regulation of Sodium Excretion:

Within the kidney, dopamine is synthesized locally in the proximal tubules. Levodopa (L-DOPA) in the glomerular filtrate is taken up by the nephron

epithelium and decarboxylated to dopamine by the aromatic amino acid decarboxylase. Majority of this conversion takes place in proximal tubular segment of the nephron (24, 93, 94, 102). Dopamine has both natriuretic and diuretic effects. Dopamine mediated inhibition of proximal tubular sodium reabsorption is a major contributor for the natriuretic effect of endogenously synthesized dopamine (35, 121, 135, 136). The natriuretic effect of dopamine is more prominent in euvolemic and volume expanded states and during sodium loading. In fact, dopamine during moderate sodium loading (5% of body weight) is responsible for approximately 50% of the increase in sodium excretion (105). Although a majority of renal dopamine is synthesized in the proximal tubules, it can act at multiple sites along the nephron. It promotes natriuresis by inhibiting sodium transport by an autocrine effect on proximal tubules and a paracrine effect at other segments like the medullary thick ascending limb and cortical collecting ducts (71, 199). However, its proximal tubular effects have drawn greater attention because most of the filtered sodium load (~70%) is reabsorbed in proximal tubules (147).

2.3 Dopamine receptors and signal transduction:

2.3.1 Classification of dopamine receptors:

Dopamine acts via the rhodopsin-like family of G protein coupled receptors (GPCR). Dopamine receptors are classified into two types, D1-like and D2-like

receptors (124). The D1-like receptors are further classified into two subtypes, D1 and the D5 receptors (named $D1_A$ and $D1_B$ respectively, in rodents). Both D1 and D5 subtypes are positively coupled to adenylyl cyclase (AC) via G_{s_α} and the D1 subtype is additionally also coupled to phospholipase C via $G_{q/11_\alpha}$. The D2-like receptors are further classified into D2, D3 and D5 subtypes (115). All the D2 like receptors are coupled negatively to AC via G_i (121). Both dopamine D1-like and D2-like receptors are expressed in the kidney. Renal dopamine receptors, like other peripheral dopamine receptors, display lower affinity (Kd) than striatal (brain) dopamine receptors (6, 7, 11). The inhibitory effect of dopamine on sodium reabsorption is primarily mediated via the D1-like receptors (135).

2.3.2 Renal dopamine D1-like receptors:

2.3.2.1 Distribution in the kidney:

Renal D1-like receptors are expressed to a greater extent in the renal cortex than in the medulla (135). D1-like receptors are located in all nephron segments including proximal convoluted tubule, pars recta, loop of Henle, distal convoluted tubule, juxtaglomerular cells, cortical and medullary collecting tubules (204). The proximal tubular segment displays the highest density of D1-like receptors compared to other tubular segments as well as the renal cortical vasculature (6-11, 204). However, glomeruli do not express any D1-like receptors (10). In the proximal

tubules, D1-like receptors are expressed on both the luminal and basolateral membranes (70).

2.3.2.2 Signal transduction in proximal tubules:

The inhibitory effect of dopamine on sodium transport at both the luminal and basolateral side is mediated by the D1-like receptors (135). At the luminal side, activation of D1-like receptors coupled to G_{s_α} leads to activation of adenylyl cyclase and increased cAMP levels and subsequent activation of cAMP dependent protein kinase (PKA) (34, 65, 68). PKA mediated phosphorylation of NHE3 results in inhibition of NHE3 activity (34, 108). In addition, a cAMP independent and G-protein dependent mechanism for inhibition of luminal NHE3 has been proposed (66).

At the basolateral side, activation of D1-like receptors coupled to $G_{q/11_\alpha}$ leads to activation of phospholipase C (PLC) and generation of inositol tris phosphate (IP3) and diacylglycerol (DAG) (67, 69). DAG mediated activation of protein kinase C (PKC) leads to phosphorylation and inhibition of the basolateral Na^+/K^+ ATPase (36, 135). It is possible that dopamine inhibits basolateral Na^+/K^+ ATPase indirectly via 20-hydroxyeicosatetraenoic acid (20-HETE) (167, 195, 196). This indirect pathway involves PLC/PKC mediated activation of phospholipase A2

(PLA2) and generation of arachidonic acid that is converted to 20-hydroxyeicosatetraenoic acid (20-HETE) by cytochrome P450 (113, 167).

The above inhibitory actions of dopamine on luminal NHE3 and basolateral Na^+/K^+ ATPase decrease proximal tubular sodium reabsorption and hence increase urinary sodium excretion (13, 14, 135, 138).

2.4 Renal dopamine D1 receptor impairment in pathogenesis of hypertension:

Numerous studies, in both hypertensive animal models and humans, point towards an involvement of a defective renal dopamine D1-like receptor function in the pathogenesis of hypertension (121, 193). Dopamine mediated natriuresis is impaired in obese Zucker rats (143), spontaneously hypertensive rats (SHR) (50, 72, 112), Dahl salt-sensitive rats (DS) (56, 100, 151, 165) and old Fischer344 rats (215). The impaired dopamine receptor mediated natriuresis is associated with decreased ability of dopamine to inhibit both NHE3 and Na^+/K^+ ATPase (20, 110-112, 137, 165). This decreased ability to inhibit NHE3 and Na^+/K^+ ATPase may involve uncoupling of G proteins from D1-like receptors (28, 33, 110, 111, 114, 122, 137, 168) and/or downregulation of D1-like receptors (33, 111, 122, 210). It is also reported that disruption of $D1_A$ receptor gene in mice causes elevations in blood pressure (3).

2.5 Impaired sodium homeostasis and renal dopamine D1 receptor function in obesity associated hypertension:

2.5.1 Role of hyperinsulinemia and hyperglycemia:

A number of studies report an association between obesity and impaired sodium excretion leading to development of hypertension (96, 192). In addition, numerous studies report an association between insulin resistance, hyperinsulinemia, hyperglycemia and impaired sodium excretion that ultimately contributes to development of hypertension (57, 73-77, 127, 132, 139, 157, 189-191). However, the role of hyperinsulinemia as a primary mediator in impairment of sodium excretion and development of hypertension is unresolved (95, 97). High fat diet causes obesity, insulin resistance, sodium retention and blood pressure elevations in dogs. In contrast, hyperinsulinemia induced in dogs by insulin infusion did not cause sodium retention or elevate blood pressure (95, 98, 99). The apparent difference between these two studies might be due to insulin resistance or some other factor that is present in obese dogs but not in normal dogs (97). However, hyperinsulinemia induced by insulin infusion increases blood pressure in Sprague Dawley rats (2, 41, 43). In these rats, Brands et al (40, 41, 43) report that there is no sodium retention associated with the increase in blood pressure using insulin infusion (1-1.5 mU/kg/min) for upto one week. In contrast, Banday and Lokhandwala (2) report that insulin infusion (5-7

mU/kg/min) in these rats for three weeks impairs sodium excretion and inhibition of proximal tubular Na^+/K^+ ATPase in response to dopamine D1 receptor agonists. The differences between the studies in dogs and rats can be attributed to species differences. Further, the differences between studies in rats can be attributed to the duration and magnitude of dosing. Most importantly, the fact that Brands et al (40, 41, 43) did not notice an increase in sodium excretion, despite the increase in blood pressure, might indicate increased sodium reabsorption (97). The increasing blood pressure and accompanying pressure natriuresis compensate for the increased sodium reabsorption and achieve equilibrium to maintain sodium balance (97). This compensation by pressure natriuresis may diminish with chronic exposure to hyperinsulinemia and/or other factors and contribute to further elevation of blood pressure (97). Therefore, insulin mediated increase in tubular sodium reabsorption might play a pivotal role in the development of obesity-associated hypertension (2, 89, 97).

Increased renal tubular reabsorption of sodium is observed in $Lepr^{fa/fa}$ (obese) Zucker rats that are used as a model to study obesity, diabetes and hypertension (4, 16). Homozygous $Lepr^{fa/fa}$ Zucker rats become overtly obese by 5-6 weeks of age (4, 16). Our lab has shown that dopamine D1 receptor mediated natriuresis and inhibition of both NHE3 and Na^+/K^+ ATPase is impaired in adult obese Zucker rats (110, 111, 137, 143). Furthermore, this defect may be due to downregulation of dopamine D1 receptors and their reduced coupling to G

proteins (111, 137). Since hyperinsulinemia (3-5 weeks of age) and hyperglycemia (6-8 weeks of age) precede hypertension, it was hypothesized that reversing these conditions could restore dopamine D1 receptor function (210). Umrani et al. (210) have reported that treating the obese Zucker rats with an insulin sensitizer (rosiglitazone) reduces insulin levels, normalizes blood pressure, restores euglycemia and restores both inhibition of Na^+/K^+ ATPase by dopamine and dopamine D1 receptor number at the membrane. Trivedi et al (208, 209) have confirmed that natriuresis in response to dopamine D1 receptor stimulation is impaired in these rats and rosiglitazone treatment restores the natriuretic response by restoring receptor-G protein coupling.

Phosphorylation of G protein-coupled receptors (GPCRs), including dopamine D1 receptors, by G protein-coupled receptor kinases (GRKs) causes uncoupling of GPCRs from G proteins as well as receptor downregulation (174). Hyperphosphorylation of dopamine D1 receptors may contribute to their defective coupling to G proteins as well as their downregulation observed in obese Zucker rats. Rosiglitazone restores dopamine D1 receptor function by reducing its hyperphosphorylation (208). GRK2 and GRK4 are potential mediators of dopamine D1 receptor hyperphosphorylation. GRK4 expression is increased in obese Zucker compared to their lean counterparts and rosiglitazone prevents this increase in expression (208). While GRK2 expression is similar in both obese and lean Zucker rats, its translocation to the membrane is increased in obese

Zucker rats (208). Rosiglitazone treatment reverses this membrane translocation of GRK2 in obese rats (208). Since rosiglitazone reverses the hyperinsulinemia and hyperglycemia observed in obese rats, it is possible that hyperinsulinemia and hyperglycemia are in part responsible for uncoupling of dopamine D1 receptors from G proteins and loss of dopamine D1 receptor mediated natriuresis and inhibition of Na^+/K^+ ATPase in these rats. Studies in other animal models and cell culture also support the role of hyperglycemia (42, 142) and hyperinsulinemia (2, 25, 26, 29) in impairment of dopamine D1 receptor function, decreased sodium excretion and development of hypertension.

While these studies provide evidence for a strong association between hyperinsulinemia and development of dopamine D1 receptor function leading to development of hypertension, role of other factors cannot be discounted. For example, insulin sensitizers (pioglitazone and troglitazone) apart from lowering insulin levels can also reduce oxidative stress (59, 83). It is possible that the blood pressure lowering effects of insulin sensitizers are secondary to lowering of oxidative stress and not necessarily due to reversal of hyperinsulinemia (59, 83).

2.5.2 Role of oxidative stress and redox sensitive transcription factor nuclear factor κ B (NFκB):

Oxidative stress results from an imbalance between the generation of reactive oxygen species (ROS, like $\bullet O_2^-$, $\bullet OH$, $\bullet ONOO^-$, H_2O_2) and their scavenging by

antioxidant mechanisms (88). Sources for ROS generation include but are not limited to mitochondria, NAD(P)H oxidases, xanthine oxidases, and uncoupled nitric oxide synthases (88). Antioxidant mechanisms may involve the conversion of highly reactive superoxide ($\bullet O_2^-$) to less reactive H_2O_2 by superoxide-dismutase, neutralization of H_2O_2 by catalases or glutathione peroxidases to water or preferential oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) to prevent oxidation of structural and functional proteins (88). Excess production of ROS and/or a decrease in activity or levels of antioxidant components can contribute to the imbalance and increased ROS levels (79, 80, 88). The resulting excess of ROS can modify structural and functional proteins, DNA and other components of the cell leading to alteration of their function and ultimately altering the cell physiology (88). In addition, ROS or their byproducts like isoprostanes (that are used as markers of oxidative stress) can themselves act as signaling molecules and contribute to alteration of cell physiology (23, 88, 153, 154). An increasing body of evidence indicates that oxidative stress plays a major role in pathogenesis of a variety of diseases including neurodegenerative diseases, cardiovascular diseases and diabetes (46, 61, 79, 80, 85, 118, 205, 214). Oxidative stress is present in both obese humans and animal models of obesity and has been implicated in the development obesity related comorbidities including hypertension (58, 82, 107, 128, 145, 159, 178, 179, 182, 198).

Obese Zucker rats also display oxidative stress (32). Treatment of obese Zucker rats with the anti-oxidant tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) reduces oxidative stress, reduces hyperinsulinemia, normalizes blood pressure, and restores renal dopamine D1 receptor mediated natriuresis and inhibition of Na^+/K^+ ATPase by restoring membrane density of renal dopamine D1 receptors and their coupling with G-proteins (32). Similarly, treatment with lipoic acid (another antioxidant) was able to decrease oxidative stress, reduce membrane translocation of GRK2 and restore renal dopamine D1 receptor mediated inhibition of Na^+/K^+ ATPase and dopamine D1 receptor-G protein coupling (31). It is difficult to draw a cause and effect relationship between oxidative stress and dopamine D1 receptor dysfunction, because tempol and other antioxidants also reverse hyperglycemia and hyperinsulinemia (21, 32).

However, other studies indicate that oxidative stress can impair dopamine D1 receptor mediated natriuresis and inhibition of Na^+/K^+ ATPase irrespective of obesity, hyperglycemia or hyperinsulinemia (18, 27, 30, 64, 144). Asghar et al. (18) have shown that oxidative stress induced by hydrogen peroxide (H_2O_2) treatment causes hyperphosphorylation of renal dopamine D1 receptors and impairment of dopamine D1 receptor mediated inhibition of Na^+/K^+ ATPase in proximal tubular cultures from Sprague Dawley rats. In addition, they showed that PKC mediated membranous translocation of GRK2 is involved in hyperphosphorylation of dopamine D1 receptor in H_2O_2 treated cultures (18).

Fardoun et al (64) have reported an impairment of dopamine D1 receptor function and coupling to G proteins in old Fischer344 rats. Further, Fardoun et al. (63) have shown that nuclear translocation of NFκB following oxidative stress, increases PKC activity leading to membranous translocation of GRK2 and subsequent dopamine D1 receptor hyperphosphorylation. Inhibiting nuclear translocation of NFκB prevents the increase in PKC activity and PKC dependent membranous translocation of GRK2 and restores dopamine D1 receptor mediated inhibition of Na^+/K^+ ATPase (63). Similarly, treatment of Sprague Dawley rats with the pro-oxidant buthionine sulfoxime (BSO) causes oxidative stress leading to impairment of dopamine D1 receptor mediated inhibition of Na^+/K^+ ATPase and natriuresis and development of salt sensitive hypertension (27, 30). This impairment is associated with increased nuclear translocation of NFκB, increased PKC activity, increased GRK2 membranous translocation, renal dopamine D1 receptor hyperphosphorylation, impaired receptor-G protein coupling and receptor downregulation (27, 30). Supplementing these pro-oxidant treated rats with the antioxidant tempol reverses the above defects and restores dopamine D1 receptor mediated inhibition of Na^+/K^+ ATPase and natriuresis and prevents the development of salt sensitive hypertension (27, 30).

Therefore, the above studies support the concept that oxidative stress may contribute to the impairment of renal dopamine D1 receptor function and

development of obesity associated hypertension. In addition, augmenting antioxidant mechanisms may provide a viable option to reduce oxidative stress, restore renal dopamine D1 receptor function and to attenuate hypertension.

2.6 Role of exercise:

Exercise is recommended as a lifestyle modification based on beneficial effects in terms of decreasing mortality and delaying the progression of a number of cardiovascular complications associated with disease states including obesity, hypertension and diabetes (1, 78, 106, 130). Exercise in both normal and overweight subjects has generally been associated with moderate decreases in blood pressure (220) improved insulin sensitivity and moderate reductions in blood glucose levels (166, 197). However, the effect of exercise on blood pressure, insulin sensitivity and blood glucose levels in both humans and animal models exhibiting metabolic syndrome is inconclusive. These studies that show either modest improvements or no effect of exercise on the above parameters have been elaborated under the discussion section along with the explanation of our results. Regardless, the amount of literature dedicated to understanding the mechanisms involved is very limited. It will be beneficial to understand the mechanisms by which exercise achieves or fails to achieve the improvements mentioned above. Specifically, the effect of exercise on renal dopamine D1 receptor function and sodium homeostasis in obesity needs to be explored.

In terms of oxidative stress, acute effect of exercise is to actually increase the production of ROS (181). Despite this, exercise reduces oxidative stress in the long term (119, 181). Since exercise can augment physiological anti-oxidant mechanisms (48, 169), it is possible that exercise mediated reductions in oxidative stress result from augmentation of antioxidant defenses (119, 181). Asghar et al (19) have shown in 23 month old Fischer344 rats that exercise augments antioxidant defenses, reduces oxidative stress and restores renal dopamine D1 receptor function. However, it is not known if exercise from an early age can prevent impairment of dopamine D1 receptor function and development of obesity and hypertension in obese Zucker rats.

2.7 Role of calorie restriction:

Restricting caloric intake to maintain energy balance is recommended as lifestyle modification based on beneficial effects in terms of decreasing mortality, increasing longevity and delaying the progression of a number of cardiovascular complications associated with disease states including obesity, hypertension and diabetes (1, 78, 130, 202). Caloric restriction in healthy non-obese individuals has been shown to decrease the risk for cardiovascular disease (134). Caloric restriction to reduce weight in hypertensive patients is associated with improvement of insulin sensitivity and a reduction of blood pressure associated with weight loss (155, 160). Recently, Metcalf et al (148) suggested that physical

inactivity is a result rather than a cause for obesity and that interventions aimed at reducing weight should focus on reducing caloric intake.

Hyperphagia in obese Zucker rats and other models of obesity, while not the sole determinant of obesity, is a major contributor to the acceleration of weight gain (16, 201). Caloric restriction in these rats leads to a decrease in weight gain (201, 225). However, even when the calories are restricted to levels comparable to lean controls, the drastic decrease in weight gain is not associated with a drastic decrease in fat accumulation relative to body weight (101, 201, 225). This might be related to a compensatory decrease in energy expenditure to favor fat storage in response to calorie restriction (101, 125).

There is disagreement regarding the effect of caloric restriction on hyperinsulinemia. While some studies report that hyperinsulinemia in these rats is responsive to caloric restriction (22, 141), others indicate that it is not responsive (52, 129, 183). The differences between these studies can be reconciled based on the level of caloric restriction and the age at which restriction is initiated. Studies employing caloric restriction at levels comparable to that of lean rats achieved a significant reduction (~50%) in insulin levels (22, 141). Further, in studies that used a lower level of restriction and did not report a significant decrease in insulin levels, a trend towards decrease was noticed (52, 183). In addition, Maddox et al (141) report that caloric restriction decreases

insulin levels only if initiated between six to twelve weeks of age. Also, maximum benefit is evident when caloric restriction is initiated at six weeks of age (141). Therefore, restricting caloric intake in obese Zucker rats to lean levels staring at an early age offers a means to reduce hyperinsulinemia.

Caloric restriction in humans, including overweight and obese subjects, has been associated with a decrease in oxidative stress (120, 200, 211). However, this beneficial effect of caloric restriction is diminished in diabetic obese subjects (200). Caloric restriction has been reported to lower oxidative stress in animal models also (39, 123, 224). However, caloric restriction in a mice model of amyotrophic lateral sclerosis paradoxically increases oxidative stress, hastens disease progression and increases mortality (173). Therefore, the effect of caloric restriction on oxidative stress can be modified by disease states and in fact be detrimental in some cases. In obese Zucker rats, we are aware of a single study reporting a decrease in blood malondialdehyde (MDA) levels (a marker of oxidative stress) with caloric restriction (129).

Literature addressing the effect of caloric restriction on blood pressure in obese Zucker rats is limited. Kurtz et al (131) reported that restricting caloric intake to lean levels in these rats for four weeks decreases weight gain and cumulative sodium retention but does not lead to decrease in blood pressure. In contrast, Maddox et al (141) reported that starting caloric restriction at an early age delays

the development of hypertension in these rats. In addition, they reported a significant correlation of blood pressure with body weight and insulin levels (141). The reason for the different observations could be that different techniques were used to measure blood pressure. While Kurtz et al (131) reported arterial blood pressure using intra-femoral catheters, Maddox et al (141) reported systolic blood pressure using tail cuff method. Both studies measured blood pressure in conscious animals. The intensity of caloric restriction or the age at which caloric restriction is initiated cannot be the reason for the differences since both started restriction at 5-6 weeks of age and employed restriction to lean levels. We are not aware of any report in these rats where the effect of caloric restriction on dopamine D1 receptor function was studied.

Therefore, it is not known if caloric restriction mediated reductions in insulin levels and/or oxidative stress can restore dopamine D1 receptor function and prevent or attenuate the development of hypertension in obese Zucker rats.

3 MATERIALS AND METHODS

3.1 STUDIES AT THREE WEEKS OF AGE:

3.1.1 PROTOCOL FOR STUDIES AT THREE WEEKS OF AGE:

Three-week-old rats were used for the experiments described below. Three-week-old rats were used because the rats are weaned at this age and they do not express the obese phenotype until five to six weeks of age. The University of Houston Animal Care and Use Committee approved all experimental protocols. Homozygous Zucker $Lepr^{fa/fa}$ **(**obese Zucker) and Zucker $Lepr^{+/+}$ (lean Zucker) rats were identified by genotyping of Zucker pups at fifteen days of age and they were weaned at nineteen days of age (Harlan Sprague Dawley Inc., Indianapolis, IN). Subsequently, the rats were obtained by the University of Houston Animal Care facility at twenty-one days of age and were maintained with a 12hr light-dark cycle and provided free access to tap water and standard rat chow containing 0.4% sodium (Labdiet® 5001, Purina Mills, St. Louis, MO). The rats were allowed two days to acclimate before initiating any experimental procedures.

3.1.2 SURGICAL PROCEDURE FOR MEASUREMENT OF BLOOD PRESSURE AND TISSUE COLLECTION:

Surgical procedures and tissue collection were performed as described earlier (49). Briefly, rats were fasted overnight and anesthetized with Inactin® (100-150 mg/Kg, i.p). Tracheotomy was performed to facilitate breathing. Right carotid artery was catheterized and following 30 min of stabilization after surgery, systolic and diastolic blood pressures were recorded using a Statham P23AC pressure transducer connected to a Grass Polygraph (model 7D, Grass Instrument Company, Quincy, MA). Mean arterial pressure (MAP) was calculated using the formula below.

$$\mathbf{MAP = [(SBP- DBP)/3] + DBP}$$

After measurement of blood pressure, blood samples were collected from carotid artery in EDTA coated tubes (BD Diagnostics, Franklin Lakes, NJ). Plasma was separated by centrifuging the blood at 1500g for 15 min at 4°C. A midline abdominal incision was made and bladder urine was collected. Blood, plasma and urine samples were stored at -80ºC until analysis. The samples were analyzed using commercially available kits and were treated appropriately before storage as directed by the kit manufacturers.

3.1.3 MEASUREMENT OF BLOOD GLUCOSE AND LIPID PROFILE: Blood glucose and lipid profile were measured using a glucose analyzer (Roche Diagnostics, Indianapolis, IN) and Cardiochek-PA® (Polymer Technology Systems, Indianapolis, IN) respectively.

3.1.4 MEASUREMENT OF FASTING PLASMA INSULIN AND INSULIN SENSITIVITY:

Insulin was measured in plasma samples by a radioimmunoassay (RIA) using a sensitive rat insulin RIA kit®, (cat. # SRI-13K, Linco Research, St. Charles, MO). Plasma samples (20μl) were incubated in glass tubes with 380μl of assay buffer (0.05M phosphosaline, pH 7.4, containing 0.025M EDTA, 0.08% sodium azide, 1% RIA grade bovine serum albumin [BSA]) and rat insulin antibody (Guinea pig anti-rat insulin serum in assay buffer) at 4°C for 24hrs. After 24hrs, ^{125}I-Insulin (specific activity 367 μCi/μg) in label hydrating buffer (assay buffer containing normal Guinea pig IgG as carrier) was added and incubated for another 24hrs at 4°C followed by addition of 1ml ice-cold precipitating reagent (Goat anti-Guinea pig IgG serum, 3% poly ethylene glycol (PEG) and 0.05% triton X-100 in 0.05M phosphosaline, 0.025M ethylene-diamine-tetra-acetic acid (EDTA), 0.08% sodium azide). The tubes were incubated for 20min at 4°C and centrifuged at 3000g for 40min at 4°C. Following centrifugation, the supernatant was decanted and pellets were used to measure radioactivity using a gamma counter (Beckman Coulter, Brea, CA). Standards (0.02-1.0ng/ml purified rat insulin), non-specific binding and total binding (B_o) of ^{125}I-Insulin were used to generate a standard curve and

used to quantify insulin in samples and expressed as ng/ml of plasma. Insulin sensitivity was assessed using quantitative insulin sensitivity check index (QUICKI) (156).

QUICKI=1/ [log (fasting insulin, μU/ml) X log (fasting glucose, mg/dl)]

3.1.5 PREPARATION OF RENAL PROXIMAL TUBULES:

3.1.5.1 Preparation of renal cortical tubular suspension:

After collecting blood and urine samples, the aorta was catheterized below the renal arteries. Celiac and superior mesenteric arteries were tied to achieve selective renal perfusion. Subsequently, the kidneys were perfused with Krebs–Henseleit buffer A (KHB-A, pH 7.4) (Table 1), maintained at 37° C with a flow rate of 4ml/min, until the kidneys were clear of blood. Concurrent with the initiation of perfusion a ligature was placed on the aorta above the kidneys to prevent re-entry of blood into the kidneys. Subsequently, the kidneys were digested in-situ by perfusing with enzyme solution (Collagenase type IV, 230U/ml and Hyaluronidase type III 250 U/ml in 40 ml KHB-A). Kidneys were excised, decapsulated, cleared of fat and connective tissue, and were weighed after blotting out excess moisture. Kidneys were cut sagitally into two equal halves and cortex was separated by cutting at the cortico-medullary junction using scissors. The cortex was chopped into fine pieces and incubated at 37° C in 20ml of enzyme solution (Collagenase type IV, 460U/ml and Hyaluronidase type III

500 U/ml in KHB-A) under 95% O_2: 5% CO_2 for 25-30 min. The suspension was filtered through a nylon mesh (mesh size 105 μm, Spectrum medical Industries, Los Angeles, CA). The filtrate was centrifuged in a swinging bucket rotor (SX4250 rotor, Beckman Coulter, Brea, CA) at 50g for 2 min to obtain a pellet which was resuspended in ice cold KHB buffer B (KHB-B, pH 7.4)(Table 1). The suspension in KHB-B was washed thrice by centrifugation at 250g for 5min and resuspended with KHB-B to remove residual enzyme solution. This *cortical tubular suspension* was used to prepare renal proximal tubules as detailed below in section 3.1.5.2.

3.1.5.2 Enrichment of renal proximal tubules:

Renal proximal tubules were enriched using 20% ficoll gradient in KHB-B. Cortical tubular suspension (15 ml) was placed as a layer over 5ml of 20% ficoll in a 50 ml centrifuge tube and centrifuged in a swinging bucket rotor (SX4250 rotor, Beckman Coulter, Brea, CA) at 4° C at 250g for 15 min. The layer resulting at the ficoll-KHB-B interface was collected and washed thrice by resuspension in KHB-C (Table 1, pH 7.4) and centrifugation at 250g for 5 minutes (Chen et al., 1993).

Table 1: ***Composition of buffers used in preparation of renal proximal tubules***

	KHB A (mM)	KHB B (mM)	KHB C (mM)
NaCl	118.0	118.0	118.0
$NaHCO_3$	27.2	27.2	27.2
KCl	4.0	4.0	4.0
$CaCl_2$	1.25	0.0	1.25
$MgCl_2$	1.2	0.12	1.2
KH_2PO_4	1.0	1.0	0.0
Glucose	5.0	5.0	5.0
HEPES	10.0	10.0	10.0

3.1.6 TEST FOR RENAL PROXIMAL TUBULAR VIABILITY:

Renal proximal tubules were tested for viability using the trypan blue exclusion test as described earlier (49). In Brief, an aliquot of renal proximal tubular suspension was incubated with trypan blue solution (0.15% in KHB-A) for 10min and placed on a glass slide. The slide was observed under a microscope at 10X magnification. At least 95% of the renal proximal tubules excluded trypan blue for the preparation to be considered viable and fit for use in further studies.

3.1.7 MEASUREMENT OF Na^+/K^+ ATPASE ACTIVITY:

Freshly prepared renal proximal tubular suspensions (1mg protein/ml) were incubated with the selective D1 receptor agonist SKF38393 (10^{-9}M, 10^{-8}M and 10^{-7} M) or vehicle (O.1% Na metabisulfite) for 15 min at 37° C. The SKF38393 or vehicle treated tubules were permeabilized by rapid freezing and thawing in a dry ice-acetone bath. To measure *total Na^+/K^+ ATPase activity,* 100 μl (1μg/ μl) of treated renal proximal tubular suspension was incubated at 37° C for 15 min in a reaction mixture (final volume 1.025 ml) containing 37.5mM imidazole, 70mM NaCl, 5mM KCl, 1mM Na EDTA, 5mM $MgCl_2$, 6mM NaN_3, 75mM Tris-HCl. The reaction was initiated using 4mM ATP. *Ouabain-insensitive ATPase activity* was measured in the presence of ouabain (1mM). The reaction was terminated by adding 50μl of ice-cold trichloroacetic acid. Activity was quantitated by measuring inorganic phosphate (Pi) colorimetrically as described by Taussky and Shorr (1953). Briefly, 150 μl of the reaction mixture was incubated at room temperature with 150 μl of ferrous sulphate-molybdate reagent (5% $FeSO_4$ in 9 ml water + 1 ml of 10% ammonium molybdate in 10N H_2SO_4) and the resulting color was spectrophotometrically measured at 740nM. *Na^+/K^+ ATPase activity* was calculated as the difference between *total and ouabain-insensitive ATPase activity.* The inhibition of Na^+/K^+ ATPase activity upon selective dopamine D1

receptor stimulation was expressed as a percentage over the basal Na^+/K^+ ATPase activity (activity in vehicle treated tubules) (Chen et al., 1993).

3.1.8 PREPARATION OF RENAL PROXIMAL TUBULAR MEMBRANES:

Renal proximal tubules were homogenized in homogenization buffer (10mM Tris HCl, 250mM sucrose, 2mM phenyl methyl sulfonyl fluoride (PMSF), Complete™ protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), pH 7.4) using a Wheaton homogenizer and centrifuged at 2500g to settle nuclear and cell debris. The supernatant was collected as the renal proximal tubular homogenate. An aliquot of the homogenate obtained in the above step was centrifuged at 38,000g for 30 min at 4° C to obtain a membrane pellet and the supernatant cytosolic fraction. The membrane pellet was resuspended in homogenization buffer and used for [^{3}H]SCH23390 binding and [^{35}S]GTPγS binding.

3.1.9 [^{3}H] SCH23390 BINDING FOR DOPAMINE D1 RECEPTOR:

Total [^{3}H] SCH23390 binding was determined by incubating membrane samples (50μg) with 20nM [^{3}H]SCH23390 (specific activity 86 Ci/mmol, Perkin Elmer, Shelton, CT) and binding buffer (50mM Tris-HCl, 2mM $MgCl_2$, 0.2 mM sodium metabisulfite, 0.2mM phenyl methyl sulfonyl fluoride (PMSF), pH 7.4) to a final volume of 250μl. Non-specific binding of [^{3}H]SCH23390 was determined separately by incorporating unlabeled SCH23390 (10μM) in the reaction mixture.

The reaction was carried out in MultiScreen-FC, 96 well filter plates with 1.2 µm FC glass fiber filters (Millipore, Billerica, MA) for 120min at 25° C. The reaction was stopped by addition of 100µl ice-cold stop solution (50mM Tris-HCl, 2mM $MgCl_2$, pH 7.4) and rapid filtration using Multiscreen Resist vacuum manifold kit (Millipore, Billerica, MA). The wells were washed four times by addition of 300 µl ice-cold stop solution followed by filtration. Filters were extracted overnight in liquid scintillation cocktail (Ready Safe Liqscint, VWR International, Suwanee, GA) and radioactivity was measured using a liquid scintillation counter (LS 6500, Beckman Coulter, Brea, CA). Specific binding was calculated as the difference between total and non-specific binding and was used to quantify dopamine D1 receptor number per mg protein.

3.1.10 [^{35}S] GTPγS BINDING:

Membrane samples (5µg), prepared as detailed above (section 3.1.9), were incubated with [^{35}S]GTPγS (final concentration of 0.6nM, specific activity 1250 Ci/mmol), in presence of SKF38393 (1nM) and guanosine diphosphate (10µM) to a final volume of 90µl in binding buffer (25mM HEPES, 15mM $MgCl_2$, 1mM EDTA, 1mM dithiothreitol, 100mM NaCl, pH 8.0). SKF38393 was excluded to determine basal [^{35}S]GTPγS binding. Unlabeled GTPγS (100µM) was incorporated in the reaction mixture to determine non-specific binding. The reaction was carried out in MultiScreen-FC, 96 well filter plates with 1.2 µm FC

glass fiber filters (Millipore, Billerica, MA) for 60min at 30° C. The reaction was terminated by addition of filtering solution (20mM Tris-HCl, 100mM NaCl and 25mM $MgCl_2$) and rapid filtration using Multiscreen Resist vacuum manifold kit (Millipore, Billerica, MA). The wells were washed four times by addition of 300 μl ice-cold filtering solution followed by filtration as above. Filters were extracted overnight in liquid scintillation cocktail (Ready Safe Liqscint, VWR International, Suwanee, GA) and radioactivity was measured using a liquid scintillation counter (LS 6500, Beckman Coulter, Brea, CA). Specific binding was calculated as the difference between total and non-specific binding and was used to quantify [^{35}S]GTPγS binding per mg protein.

3.1.11 IMMUNOBLOTTING FOR G PROTEINS:

Renal proximal tubular homogenates were supplemented with Laemmli buffer (62.5mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS) and 2.5% 2-mercapto-ethanol and bromophenol blue) to prepare samples for electrophoresis. The samples (10μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P®, Millipore, Billerica, MA). The membrane was blocked for 1hr with 5% fat free milk in phosphate buffered saline-tween (PBST) followed by incubation with anti-$G_s\alpha$-subunit rabbit polyclonal IgG or anti-$G_{q/11}\alpha$-subunit rabbit polyclonal IgG (Calbiochem, San Diego, CA) for 15hrs at 4°C. The

membrane was washed with PBST thrice for 10min each and incubated in horseradish peroxidase (HRP) linked goat anti-rabbit IgG (Santacruz Biotechnology, Santacruz, CA) for 1hr. The membrane was again washed thrice (10 min each) with PBST. G protein bands were visualized with western blotting luminol reagent® (Santacruz Biotechnology, Santacruz, CA) on X-ray film and densitometrically quantified using AlphaInnotech® imaging software (Cell Biosciences, Santa Clara, CA).

3.1.12 OXIDATIVE STRESS MARKERS:

3.1.12.1 MEASUREMENT OF PROTEIN CARBONYLATION:

Protein carbonylation was measured using OxyBlot® protein oxidation detection kit (Millipore, Billerica, MA) as per the manufacturer's protocol. Two separate aliquots of renal proximal tubular homogenate (20μg protein) were denatured by addition of equal volume of 12% sodium dodecyl sulfate (SDS). One aliquot was used for derivatization to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by incubation with 2,4-dinitrophenylhydrazine (DNPH) and the other was incubated with derivatization control solution and used as a derivatization control. The derivatization reaction was terminated after 15min by addition of 7.5μl neutralization solution to both aliquots. Both the derivatized samples and derivatization controls were subjected to SDS-PAGE and transblotted onto PVDF membranes. Membranes with transblotted proteins were blocked with 1% bovine

serum albumin (BSA) in PBST and then incubated for 1hr at 25°C with antibody specific to DNP-hydrazone. The membranes were washed three times (10min each) followed by incubation with HRP-linked antibody for 1hr at 25°C. Following washing, the derivatized proteins were visualized with western blotting luminol reagent® (Santacruz Biotechnology, Santacruz, CA) on X-ray film and densitometrically quantified using AlphaInnotech® imaging software (Cell Biosciences, Santa Clara, CA).

3.1.12.2 MEASUREMENT OF PLASMA AND URINARY 8-ISOPROSTANE:

8-isoprostane was measured by a competitive enzyme immunosorbent assay (EIA) using 8-isoprostane EIA kit® (Cayman Chemical Company, Ann Arbor, MI) as per the manufacturer's protocol. Fresh plasma and urine samples were supplemented with 0.005% butylated hydroxytoluene (BHT) and 10μM indomethacin and stored at -80°C. Urine samples were diluted 1:50 with EIA buffer provided with the kit while plasma samples were used without dilution. 8-isoprostane standards (0.8-500pg/ml) and urine and plasma samples (50μl) were incubated in a 96 well plate with 8-isoprostane-acetylcholine-esterase (AChE) tracer and 8-isoprostane specific rabbit antiserum for 18hrs at 4°C. Any unbound reagents were washed and the plate was incubated with 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) for 90min. Absorbance of the resulting color was measured at a wavelength of 410nm and used for

quantification as per kit manufacturer's recommendations. Urinary 8-isoprostane content was normalized with creatinine levels. Creatinine levels were determined as described below in section 3.2.8.

3.1.12.3 MEASUREMENT OF PROTEIN NITROSYLATION:

Samples for electrophoresis were prepared by supplementing renal proximal tubular homogenates with Laemmli buffer (62.5mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS) and 2.5% 2-mercapto-ethanol and bromophenol blue). The samples (10μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P®, Millipore, Billerica, MA). The membrane was blocked for 1hr with 1% bovine serum albumin (BSA) in phosphate buffered saline-tween (PBST) followed by incubation with HRP conjugated anti-nitrotyrosine (clone 1A6) IgG (Millipore, Billerica, MA). The protein bands were visualized with western blotting luminol reagent® (Santacruz Biotechnology, Santacruz, CA) on X-ray film and densitometrically quantified using AlphaInnotech® imaging software (Cell Biosciences, Santa Clara, CA).

3.1.12.4 MEASUREMENT OF WHOLE BLOOD GLUTATHIONE LEVELS:

Reduced (GSH) and oxidized (GSSG) glutathione were measured using BIOXYTECH® GSH/GSSG-412™ kit (OxisResearch™, Portland, OR). The kit

employs the enzymatic method described by Tietze (207). Briefly, GSSG is converted to GSH by glutathione reductase, which on reaction with 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) develops color. The rate of color development, measured spectrophotometrically at 412nm, is used to quantify GSH and GSSG levels. Two separate aliquots of whole blood were stored at -80°C and used for determination of total (GSH + GSSG) glutathione levels and GSSG alone. The aliquot used to determine GSSG levels alone was treated with 1-methyl-2-vinylpyridinium, a GSH scavenger, before storage at -80°C to prevent non-enzymatic conversion of GSH to GSSG. After thawing, both aliquots were extracted with 5% metaphosphoric acid and the resulting extracts were incubated with glutathione reductase and Ellman's reagent for 5min. The chromogenic reaction was initiated by addition of NADPH and change of absorbance was measured for 3min at 412nm wavelength. Total glutathione (GSH+GSSG) and oxidized glutathione (GSSG) levels were quantified using rate curves generated using appropriate standards. GSH levels were calculated as the difference between total glutathione and GSSG levels.

3.1.12.5 MEASUREMENT OF HEMEOXYGENASE-1 (HO-1) LEVELS:

Hemeoxygenase-1 (HO-1) levels in plasma and renal proximal tubular homogenates were measured by a sandwich enzyme linked immunosorbent assay (ELISA), using rat HO-1 ELISA kit® (Assay Designs, Ann Arbor, MI) as per

the kit manufacturer's protocol. Briefly, plasma or renal proximal tubular homogenates were incubated in 96 well plates pre-coated with anti-HO-1 mouse monoclonal antibody followed by incubation with rabbit polyclonal anti-HO-1 antibody. Subsequently, the plate was incubated with HRP-linked anti-rabbit IgG followed by incubation with tetramethylbenzidine, an HRP-substrate, to develop a blue color. Color development was stopped by addition of an acidic stop solution and the resulting yellow color was quantified spectrophotometrically by measuring absorbance at 450nm. Appropriate standards were used to quantify HO-1 levels, as ng/ml in plasma and ng/mg protein in renal proximal tubular homogenates.

3.1.12.6 MEASUREMENT OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY:

Superoxide dismutase (SOD) activity in plasma and renal proximal tubular homogenates was measured using superoxide dismutase assay kit® (Cayman Chemical, Ann Arbor, MI). Renal proximal tubules were homogenized in SOD homogenization buffer (20mM HEPES, 1mM EGTA, 210mM mannitol, 70mM sucrose, pH 7.4) followed by centrifugation at 1500g for 5min at 4°C. The resulting supernatant was used as the renal proximal tubular homogenate. Plasma samples were diluted (1:5) in sample buffer (50mM Tris-HCl, pH 8.0). Bovine Cu/Zn SOD (0.025-0.25U) was used to generate a standard curve. Standards and samples (10μl) were incubated on an orbital shaker in a 96 well

plate with 200μl of tetrazolium salt solution in assay buffer (50mM Tris-HCl, 0.1mM diethylenetriaminepentaacetic acid, 0.1mM hypoxanthine, pH 8.0) and 20μl of xanthine oxidase at room temperature for 20min. The resulting yellow color was quantified by measuring absorbance at a wavelength of 450nm. SOD activity was expressed as Units/ml for plasma samples and Units/mg protein for renal proximal tubular homogenates. One unit of SOD was described by the kit manufacturer as the amount of enzyme required to exhibit dismutation of 50% superoxide radicals.

3.1.13 MEASUREMENT OF NUCLEAR TRANSLOCATION OF NFκB:

3.1.13.1 *Separation of nuclear and cytosolic fractions:*

Renal proximal tubular nuclear and cytosolic fractions were prepared using *NE-PER™ Nuclear and Cytosplasmic Extraction Reagents* kit® (Pierce Biotechnology, IL). Renal proximal tubules were homogenized in cytoplasmic extraction reagent® (CER) I, using a glass douncer followed by incubation with ice-cold CERII for 1min. The homogenate was centrifuged at 16,000g for 5min and the supernatant was saved as the cytosolic fraction. The pellet was resuspended in ice-cold nuclear extraction reagent (NER) and incubated on ice with vortexing at regular intervals for 40min followed by centrifugation at 16,000g for 10min and the supernatant was saved as the nuclear fraction.

3.1.13.2 *Immunoblotting for NFκB:*

Samples for electrophoresis were prepared by supplementing nuclear and cytosolic fractions with Laemmli buffer (62.5mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS) and 2.5% 2-mercapto-ethanol and bromophenol blue). The samples (15μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P®, Millipore, Billerica, MA). The membrane was blocked for 1hr with 5% fat free milk in phosphate buffered saline-tween (PBST) followed by incubation with anti-NFκB-p65 subunit rabbit polyclonal IgG (Calbiochem, San Diego, CA) for 18hrs at 4°C. The membrane was washed with PBST thrice for 10 min each and incubated in horseradish peroxidase (HRP) linked goat anti-rabbit IgG (Santacruz Biotechnology, Santacruz, CA) for 1hr. The membrane was again washed thrice (10 min each) with PBST and protein bands were visualized with western blotting luminol reagent® (Santacruz Biotechnology, Santacruz, CA) on X-ray film and densitometrically quantified using AlphaInnotech® imaging software (Cell Biosciences, Santa Clara, CA).

3.2 PROTOCOL FOR EXERCISE STUDY:

3.2.1 ANIMALS AND EXERCISE PROTOCOL:

Homozygous Zucker Lepr$^{fa/fa}$ **(**obese Zucker) and Zucker Lepr$^{+/+}$ (lean Zucker) rats were identified by genotyping of Zucker pups at fifteen days of age and they were weaned at nineteen days of age (Harlan Sprague Dawley Inc., Indianapolis, IN). Subsequently, the rats were obtained by the University of Houston Animal Care facility at twenty-one days of age and were maintained with a 12hr light-dark cycle and provided free access to tap water and standard rat chow containing 0.4% sodium (Labdiet® 5001, Purina Mills, St. Louis, MO). The rats were allowed two days to acclimate before initiating any experimental procedures. The rats were randomly divided into four groups; lean sedentary, lean exercised, obese sedentary and obese exercised group. Lean exercised group was excluded from some studies specified below. The exercise group was subjected to treadmill exercise for eight weeks according to a previously reported protocol with some modifications (84). Briefly, the rats were exercised at 0º incline and a speed of 10 m/min for 1hr daily (5min breaks every 15min), five days a week. Body weights were recorded at the beginning of the study and weekly thereafter until the end of the study. The rats were placed in metabolic cages to measure food and water intake from fifth to eighth week of age. After eight weeks of exercise, at eleven weeks of age, the rats were used for the

experiments described below. Before any further experimental procedures were performed, the rats were rested for 48hrs after the last exercise session to eliminate interference from acute effects of exercise.

3.2.2 SURGICAL PROCEDURE FOR MEASUREMENT OF BLOOD PRESSURE AND TISSUE COLLECTION:

Surgical procedures were performed as described in section 3.1.2. Heart, liver, spleen, visceral fat and brain were cleared of connective tissue and weighed after blotting out excess moisture. Renal proximal tubules were prepared as described in section 3.1.5 above. The organs and tissues were stored at -80°C.

3.2.3 MEASUREMENT OF BLOOD GLUCOSE AND LIPID PROFILE:

Blood glucose and lipid profile were measured as described in section 3.1.3.

3.2.4 MEASUREMENT OF FASTING PLASMA INSULIN AND INSULIN SENSITIVITY:

Fasting plasma insulin and insulin sensitivity were measured as described in section 3.1.4.

3.2.5 OXIDATIVE STRESS MARKERS:

3.2.5.1 MEASUREMENT OF URINARY 8-ISOPROSTANE:

Urinary 8-isoprostane was measured as described above in section 3.1.12.2.

3.2.5.2 MEASUREMENT OF MALONDIALDEHYDE (MDA) LEVELS:

Malondialdehyde (MDA) levels were measured as thiobarbituric acid reactive substances (TBARS) using the method of Mihara and Urchiyama (150). Renal proximal tubules were homogenized in ice-cold 1.15% KCl, (pH 7.4) with a polytron homogenizer. The homogenate was centrifuged at 12000g for 5min and the supernatant was used for measurement of TBARS. A 1ml aliquot of the above supernatant (protein conc. 2mg/ml) was added to 2ml solution of 0.25N HCl, 15% trichloro-acetic acid and 0.375% 2-thiobarbituric acid. The reaction mixture was heated for 15 min in boiling water and after cooling was extracted with isobutanol. Absorbance of the isobutanol extract was measured spectrophotometrically at 535nm and quantified using the molar extinction coefficient of MDA (1.56×10^5 M^{-1} cm^{-1}) and expressed as nmoles MDA/mg of protein.

3.2.5.3 MEASUREMENT OF WHOLE BLOOD GLUTATHIONE LEVELS:

Total glutathione levels were measured in whole blood samples as described above in section 3.1.12.4.

3.2.5.4 MEASUREMENT OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY:

Plasma and renal proximal tubular SOD activity was measured as described in section 3.1.12.6.

3.2.6 MEASUREMENT OF URINARY ALBUMIN EXCRETION:

Urinary albumin was measured by an enzyme immuno-assay (EIA) using rat albumin EIA kit® (Cayman Chemical Company, Ann Arbor, MI) as per the manufacturer's protocol. Briefly, 50μl of diluted urine samples (1:5000 in EIA buffer®) were incubated with rat albumin acetylcholine esterase (AChE) tracer® and rat albumin antiserum in a 96 well plate for 20hrs at 4°C. At the end of the incubation period, the plate was washed 5 times with wash buffer and 200μl of Ellman's reagent was added to the wells to develop a yellow color. The color was quantified by measuring absorbance at a wavelength of 410nm. Standards (7.8-1000ng/ml rat albumin), non-specific binding and total binding (B_o) of AChE tracer were used to generate a standard curve and to determine albumin concentrations (ng/ml). Albumin concentrations were normalized with creatinine levels and expressed as μg/mg of creatinine. Creatinine levels were measured as described below in section 3.2.8.

Since there were no differences in measures of oxidative stress and other physiological parameters between the lean sedentary and lean exercised groups, lean exercised group was eliminated from the following studies.

3.2.7 MEASUREMENT OF NATRIURETIC RESPONSE TO SELECTIVE DOPAMINE D1 RECEPTOR AGONIST (SKF38393):

A separate set of rats was used for the measurement of natriuretic response to SKF38393. Rats were fasted overnight and anesthetized with Inactin® (100 mg/Kg, i.p). Tracheotomy was performed to facilitate breathing and right carotid artery was catheterized with PE-50 tubing to measure blood pressure (as described earlier) and to collect blood. The left jugular vein was catheterized using PE-50 tubing for infusing saline or drug. Normal saline was infused continuously throughout the experimental period to maintain stable urine output and to prevent dehydration of rats. For collecting urine, following midline laparotomy, the left ureter was catheterized with PE-10 tubing connected to tygon® tubing.

Following a stabilization period of 45 minutes after initiation of saline infusion, urine and blood samples were collected for 5 consecutive 30 min periods (C1, C2, D, R1 and R2). The five consecutive collection periods are as follows:

a. Two basal collection periods (basal 1, basal 2), during which saline alone was infused.
b. Drug period, during which SKF 38393 (3μg/kg/min in saline) was infused.

c. Two recovery periods (R1 and R2), during which saline alone was infused.

Plasma was separated by centrifuging the blood samples at 1500g for 15 minutes at 4°C. Sodium and creatinine concentrations were measured in both plasma and urine samples. Sodium concentrations were measured using a flame photometer (Model 2655-10, Cole Parmer Instrument Company, IL). Creatinine concentrations were measured using creatinine assay kit® (BioVision, Mountain View, CA), as described below in section 3.2.8. Creatinine clearance ($CL_{creatinine}$) was calculated using the formula given below and considered as a marker of glomerular filtration rate (GFR). Urinary sodium excretion ($U_{Na}V$) and fractional excretion of sodium (FE_{Na}) were calculated using the formulae given below.

$$FE_{Na} = \frac{U_{Na}\,V\ (\mu mol/min) \times 100}{\text{Plasma Na concentration } (\mu mol/\mu l) \times GFR\ (\mu l/min)}$$

$$U_{Na}\,V\ (\mu mol/min) = \text{Urine flow } (\mu l/min) \times \text{Urinary sodium concentration } (\mu mol/\mu l)$$

$$CL_{creatinine} = \frac{\text{Urine flow (ml/min)} \times \text{Urine creatinine concentration (mg/dl)}}{\text{Plasma creatinine concentration (mg/dl)}}$$

3.2.8 MEASUREMENT OF CREATININE LEVELS:

Creatinine was measured by a colorimetric assay using creatinine assay kit® (BioVision, Mountain View, CA) as per the kit manufacturer's protocol. The assay involves enzymatic conversion of creatinine to creatine and creatine to sarcosine. The oxidation product of sarcosine reacts with a probe® to generate color that was quantified by measuring absorbance at 570nm. Urine (1:100) and plasma (1:25) samples were diluted with assay buffer. Diluted samples and creatinine standards (2-10 nmoles) were incubated in a 96 well plate with the assay buffer®, creatininase, creatinase, enzyme mix® and probe®. The plate was incubated for 1hr at 37°C and absorbance was measured at 570nm and used to quantify creatinine content using the standard curve.

3.2.9 PREPARATION OF RENAL PROXIMAL TUBULES:

Renal proximal tubules were prepared as described in section 3.1.5.

3.2.10 TEST FOR RENAL PROXIMALTUBULAR VIABILITY:

Renal proximal tubules were tested for viability as described in section 3.1.6.

3.2.11 PREPARATION OF RENAL PROXIMAL TUBULAR MEMBRANES:

Renal proximal tubular membranes were prepared as described in section 3.1.8.

3.2.12 [^{35}S] GTPγS BINDING:

[^{35}S] GTPγS binding was measured in renal proximal tubular membranes as described in section 3.1.10 except that three different concentrations (1, 10 and 100nM) of SKF38393 were used.

3.2.13 MEASUREMENT OF NUCLEAR TRANSLOCATION OF NFκB:

Nuclear translocation of the redox sensitive transcription factor NFκB was measured using the procedure described under section 3.1.13.

3.3 PROTOCOL FOR CALORIC RESTRICTION STUDY:

3.3.1 ANIMALS AND CALORIC RESTRICTION PROTOCOL:

Homozygous Zucker Lepr$^{fa/fa}$ **(**obese Zucker) rats were obtained at five weeks of age and were maintained with a 12hr light-dark cycle and provided free access to tap water and standard rat chow containing 0.4% sodium (Labdiet® 5001, Purina Mills, St. Louis, MO). The rats were allowed seven days to acclimate before initiating any experimental procedures. After acclimation, the rats were placed in metabolic cages and food and water intake was recorded daily for seven days to get a baseline reading. At seven weeks of age, the rats were randomly divided into two groups, ad libitum group and caloric restriction group. The ad libitum group was provided free access to standard rat chow and caloric restriction group was given 50% of the amount consumed by the ad libitum group. Both

groups were given free access to water. After eight weeks of caloric restriction (at 15 weeks of age), blood samples were collected from the tail vein to measure insulin levels. Fasting plasma insulin levels were measured as described below in section 3.3.4. After ten weeks of caloric restriction (at 17 weeks of age), natriuretic response to SKF38393 and oxidative stress markers were measured as described below.

3.3.2 SURGICAL PROCEDURE FOR MEASUREMENT OF BLOOD PRESSURE AND TISSUE COLLECTION:

Surgical procedures were performed as described in section 3.1.2. Liver, spleen, visceral fat and heart were cleared of connective tissue and weighed after blotting out excess moisture. Renal cortical homogenates were prepared as described in section 3.1.5.1 above. The organs and renal cortical homogenates were stored at -80°C.

3.3.3 MEASUREMENT OF BLOOD GLUCOSE AND LIPID PROFILE:

Blood glucose and lipid profile were measured as described in section 3.1.3.

3.3.4 MEASUREMENT OF FASTING PLASMA INSULIN AND INSULIN SENSITIVITY:

Both ad libitum and caloric restriction group were fasted overnight and the tail vein was transiently catheterized with Surflo Winged Infusion Set™ 3.5 21gx3/4"

(Henry Schein, Melville, NY) to collect blood. The needle puncture was sealed with 3M-Vetbond™ 1469-SB (Henry Schein, Melville, NY) and betadine solution (Henry Schein, Melville, NY) was applied to the puncture area. Plasma was separated by centrifuging the blood at 1500g for 15 min at 4°C. Fasting plasma insulin and insulin sensitivity were measured as described in section 3.1.4.

3.3.5 OXIDATIVE STRESS MARKERS:

3.3.5.1 MEASUREMENT OF PROTEIN CARBONYLATION:

Protein carbonylation was measured using renal cortical homogenates as described in section 3.1.12.1.

3.3.5.2 MEASUREMENT OF PLASMA AND URINARY 8-ISOPROSTANE:

Plasma and urinary 8-isoprostane were measured as described above in section 3.1.12.2.

3.3.5.3 MEASUREMENT OF PROTEIN NITROSYLATION:

Renal cortical protein nitrosylation was measured by a competitive enzyme linked immunosorbent assay (ELISA) using a nitrotyrosine assay kit® (Millipore, Billerica, MA) according to the kit manufacturer's protocol. White, high binding 96 well plates were incubated with 100μl of nitrated BSA solution (5μg/ml) in carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.5) for 20hrs at 4°C. The

plates were washed twice with Tween-20 supplemented tris-buffered-saline (TBS-T, 50mM Tris-HCl, 150mM NaCl, 0.05% Tween-20, pH 7.4) followed by washing with tris-buffered saline (TBS, 50mM Tris-HCl, 150mM NaCl, pH 7.4). The plate was incubated with 150μl of blocking buffer® for 1hr at 37°C to block nonspecific binding sites. After discarding the blocking buffer, 50μl of renal cortical homogenates (10μg/ml) or standards (0.1-1200μg/ml) were incubated with 50μl of anti-nitrotyrosine rabbit IgG for 1hr at 37°C. The plate was washed once with TBS-T and thrice with TBS. HRP-conjugated anti-Rabbit IgG (100μl) was added to wells and the plate incubated for 1hr at 37°C. The plate was again washed once with TBS-T and thrice with TBS followed by addition of 75μl of LumiGLO® chemiluminescent substrate. After incubation for 10min, the resulting luminescence was measured using a plate reader and used to quantify nitrotyrosine levels as pmoles/mg of protein.

3.3.5.4 MEASUREMENT OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY:

Plasma and renal cortical SOD activity was measured as described in section 3.1.12.6.

3.3.5.5 MEASUREMENT OF MALONDIALDEHYDE (MDA) LEVELS:

Malondialdehyde (MDA) levels were measured in renal cortical homogenates as described in section 3.2.5.2.

3.3.6 MEASUREMENT OF NATRIURETIC RESPONSE TO SELECTIVE D1 RECEPTOR AGONIST (SKF38393):

Natriuretic response to the selective dopamine D1 receptor agonist SKF38393 was measured as described in section 3.2.7. Sodium was measure using atomic absorption spectroscopy (AAnalyst 400 atomic absorption spectrometer, Perkin Elmer, Waltham, MA).

4. RESULTS:

4.1 STUDIES AT THREE WEEKS OF AGE:

The studies described in this section were performed in three-week-old Lepr$^{fa/fa}$ Zucker (obese) rats and Lepr$^{+/+}$ Zucker (lean) controls. Three-week-old Lepr$^{fa/fa}$ Zucker (obese) rats and Lepr$^{+/+}$ Zucker (lean) controls were identified by genotyping as they cannot be visually differentiated at this age.

4.1.1 General physiological parameters of Lepr$^{fa/fa}$ Zucker (obese) and Lepr$^{+/+}$ Zucker (lean) rats at three weeks of age:

At three weeks of age, Lepr$^{fa/fa}$ Zucker (obese) rats cannot be visually differentiated from the Lepr$^{+/+}$ Zucker (lean) controls and are of similar weight as their lean counterparts (Table 2). Kidney weight to body weight ratio was similar in both the groups (Table 2). Three weeks old Lepr$^{fa/fa}$ Zucker (obese) rats display significantly higher levels of total cholesterol (Table 2), HDL cholesterol (Table 2) and triglycerides (Table 2). Mean arterial pressure in Lepr$^{fa/fa}$ Zucker (obese) rats at three weeks of age is not significantly different from Lepr$^{+/+}$ Zucker (lean) controls (fig. 1A). Fasting blood glucose levels are similar in both the groups (fig. 1B). However, Lepr$^{fa/fa}$ Zucker (obese) rats display significantly higher fasting plasma insulin levels (~2 fold) and decreased insulin sensitivity

(measured using quantitative insulin check index, QUICKI) compared to their lean counterparts (fig. 1C and D respectively).

4.1.2 Levels of oxidative stress markers at three weeks of age:

4.1.2.1 Proximal tubular protein carbonylation:

Three weeks old $Lepr^{+/+}$ Zucker (lean) and $Lepr^{fa/fa}$ Zucker (obese) rats displayed similar levels of renal proximal tubular protein carbonylation (fig. 2). Since no bands were detected for derivatization controls in both the groups (fig. 2, upper panel, lanes 1 and 3), the entire strip of protein bands was used for quantification.

4.1.2.2 Plasma and urinary 8-isoprostane:

$Lepr^{+/+}$ Zucker (lean) and $Lepr^{fa/fa}$ Zucker (obese) rats displayed similar levels of both plasma and urinary 8-isoprostane (fig. 3A and 3B respectively).

4.1.2.3 Proximal tubular protein nitrosylation:

Protein nitrosylation levels in renal proximal tubules were similar in both $Lepr^{+/+}$ Zucker (lean) and $Lepr^{fa/fa}$ Zucker (obese) rats at three weeks of age (fig. 4).

4.1.2.4 Whole blood glutathione levels:

Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats displayed similar levels of both reduced glutathione (GSH) and oxidized glutathione (GSSG) (fig. 5A) and hence the ratio of GSH to GSSG was also similar (fig. 5B).

4.1.2.5 Plasma and renal proximal tubular hemeoxygenase-1 levels:

Plasma hemeoxygenase-1 (HO-1) levels were higher in Lepr$^{fa/fa}$ Zucker (obese) rats (fig. 6A) but proximal tubular HO-1 levels were similar in both the groups (fig. 6B).

4.1.2.6 Plasma and renal proximal tubular superoxide dismutase activity:

Plasma superoxide dismutase (SOD) activity was similar in both the groups (fig. 7A). However, SOD activity in proximal tubular homogenate was significantly decreased in Lepr$^{fa/fa}$ Zucker (obese) compared to Lepr$^{+/+}$ Zucker (lean) rats (fig. 7B).

4.1.3 Inhibition of renal proximal tubular Na^+/K^+ ATPase activity by SKF38393 at three weeks of age:

At three weeks of age, SKF38393 inhibited renal proximal tubular Na^+/K^+ ATPase activity in a dose dependent manner in Lepr$^{+/+}$ Zucker (lean) rats but failed to do

so in $Lepr^{fa/fa}$ Zucker (obese) rats (fig. 8A). Basal Na^+/K^+ ATPase activity was found to be similar in both the groups (8B).

4.1.4 Renal proximal tubular dopamine D1 receptor-G protein coupling at three weeks of age:

Renal dopamine D1 receptor-G protein coupling, measured as stimulation of GTPγS binding in response to SKF38393, was significantly lower in $Lepr^{fa/fa}$ Zucker (obese) rats when compared to $Lepr^{+/+}$ Zucker (lean) rats (fig. 9A). Basal GTPγS binding (fig. 9B), and protein levels of the G proteins $G_{S\alpha}$ (fig. 10A) and $G_{q/11\alpha}$ (fig. 10B) were similar in both the groups.

4.1.5 Renal proximal tubular membrane Dopamine D1 receptor levels at three weeks of age:

Renal proximal tubular membrane dopamine D1 receptor levels, measured using [^{3}H]SCH23390 binding, were similar in both the $Lepr^{+/+}$ Zucker (lean) and $Lepr^{fa/fa}$ Zucker (obese) rats at three weeks of age (fig. 11).

4.1.6 Nuclear translocation of NFκB in renal proximal tubules at three weeks of age:

Nuclear and cytosolic levels of NFκB in Lepr$^{fa/fa}$ Zucker (obese) were similar to those in Lepr$^{+/+}$ Zucker (lean) rats (fig. 12). In both groups, nuclear levels of NFκB were lower than cytosolic levels (fig 12).

4.2 EFFECT OF EXERCISE:

It has been suggested that starting a lifestyle intervention early in life might alter physiological processes that might be more difficult to alter at a later age. Hence, starting a lifestyle intervention at an early age could be more beneficial (130). Therefore, we subjected the rats to exercise from three weeks of age, for eight weeks, until the age of eleven weeks. The results described in this section are from studies performed at eleven weeks of age after eight weeks of exercise period.

4.2.1 Effect of exercise on general physiological parameters: Obese rats consumed significantly higher amounts of food and water when compared to lean rats (fig. 13A and B respectively) from five to eight weeks of age. Exercise did not alter the intake of either food or water in both lean and obese rats (fig. 13A and B respectively). Obese rats displayed significantly higher body weights compared to lean rats from six weeks of age until eleven weeks of age (fig. 13C). Exercise

did not reduce the gain in body weight in both obese and lean rats (fig. 13C). After completion of exercise or sedentary protocol, obese sedentary rats displayed significantly higher blood pressure, fasting blood glucose levels, profound hyperinsulinemia (~9 fold) and decreased insulin sensitivity (QUICKI) when compared to lean rats (fig. 14 A, B, C and D respectively). Exercise had no effect on blood pressure, fasting blood glucose levels, insulin levels or insulin sensitivity in both lean and obese rats (fig. 14 A, B, C and D respectively).

Triglyceride levels were significantly higher (~6 fold) in obese rats compared to lean rats (Table 3). Exercise significantly reduced triglyceride levels in obese rats but not lean rats (Table 3). Total cholesterol levels were similar in both obese sedentary and exercised groups (Table 3). Total cholesterol levels were undetectable, using the method employed, in lean rats. HDL cholesterol was significantly higher in obese rats compared to lean rats (Table 3). While exercise did not affect HDL cholesterol levels in lean rats, it paradoxically reduced HDL cholesterol in obese rats (Table 3). However, the levels still remained significantly higher compared to lean rats (Table 3). Obese rats compared to lean rats, display a significantly higher body weight and fat to body weight ratio (Table 3). Exercise did not alter the ratio in lean rats but paradoxically increased the ratio in obese rats by ~15% (Table 3).

Kidneys, heart, spleen and brain of obese rats showed significantly lower organ weight to body weight ratios compared to lean rats and exercise did not alter this. Liver to body weight ratios were similar in all the groups (Table 3).

4.2.2 Effect of exercise on oxidative stress:

4.2.2.1 Urinary 8-Isoprostane:

Obese rats display increased levels of urinary 8-Isoprostane when compared to lean rats (fig. 15). Exercise decreased levels of urinary 8-Isoprostane in obese rats but did not affect levels in lean rats (fig. 15).

4.2.2.2 Renal malondialdehyde (MDA):

Obese rats display increased levels of renal MDA than lean rats (fig. 16). Exercise decreased levels of MDA in obese rats but did not affect levels in lean rats (fig. 16).

4.2.2.3 Whole blood glutathione:

Obese rats display decreased blood glutathione levels and exercise did not cause any changes in glutathione levels in both lean and obese rats (fig. 17).

4.2.2.4 Plasma and proximal tubular superoxide dismutase (SOD):

Obese rats also showed decreased superoxide dismutase (SOD) activity in both plasma (fig. 18A) and proximal tubular homogenates when compared to lean rats

(fig. 18B). Exercise augmented SOD activity in both plasma (fig. 18A) and proximal tubular homogenates (fig. 18B) of obese rats while the lean exercised group did not show any significant changes.

4.2.3 Effect of exercise on urinary albumin excretion:

Obese rats displayed ~20 fold higher urinary albumin excretion when compared to lean rats (fig. 19). Exercise decreased urinary albumin excretion by ~50% in obese rats but did not have any affect on lean rats (fig. 19).

Since exercise did not change any of the above parameters tested in lean rats, the lean exercised group was excluded from the following studies.

4.2.4 Effect of exercise on natriuretic response to SKF38393: Intravenous infusion of SKF38393 (3μg kg^{-1}.body wt. min^{-1}) increased urine flow, urinary sodium excretion ($U_{Na}V$) and fractional excretion of sodium (FE_{Na}) in lean rats but not in obese rats (fig. 20, 21 and 22 respectively). Exercise increased diuresis in response to SKF38393 (fig. 20). However, it failed to restore the natriuretic response in obese rats since both urinary sodium excretion ($U_{Na}V$) and FE_{Na} did not increase in response to SKF38393 infusion (fig. 21 and 22 respectively). Basal glomerular filtration rate (GFR) was similar in all the groups and SKF38393 infusion did not alter GFR (fig. 23), blood pressure or heart rate. Lean rats showed a marginal decrease in GFR during recovery phase (fig. 23).

4.2.5 Effect of exercise on SKF38393 induced dopamine D1 receptor-G protein coupling: SKF38393 (1, 10 and 100nM) induced a concentration dependent increase in [^{35}S]GTPγS binding in proximal tubular membranes from lean rats (fig. 24A). However, SKF38393 failed to elicit any significant [^{35}S]GTPγS binding in both the sedentary and exercised obese rats (fig. 24A). Thus, obese rats display defective dopamine D1 receptor-G protein coupling and exercise fails to correct this defect in coupling. Basal [^{35}S]GTPγS binding was similar in all the groups (fig. 24B) (lean sedentary, 229.0±9.32 fmoles/mg; obese sedentary, 259.1±16.92 fmoles/mg; obese exercised, 264.5±10.48 fmoles/mg).

4.2.6 Effect of exercise on nuclear translocation of nuclear factor κ B (NFκB): Significantly higher levels of the redox sensitive transcription factor NFκB (p65) were observed in proximal tubular nuclear extracts of obese rats compared to lean rats (fig. 25). Exercise significantly decreased the nuclear content of NFκB (fig. 25). The decrease in nuclear content of NFκB was accompanied with a corresponding increase in the cytosolic content indicating that exercise reduced the nuclear translocation of NFκB in obese rats (fig. 25).

4.3 EFFECT OF CALORIC RESTRICTION:

The results described below are from studies conducted in homozygous Zucker Lepr$^{fa/fa}$ (obese Zucker) rats that were either provided ad-libitum access to food

or were calorie restricted. Calorie restricted group was provided 50% of the food consumed by ad-libitum from seven weeks of age for ten weeks (seventeen weeks of age).

4.3.1 Effect of caloric restriction on general physiological parameters: Ad-libitum group consumed approximately 40-45 grams of food from 7 weeks of age until seventeen weeks of age (fig. 26A). Caloric restriction group was provided half the amount of food consumed by ad-libitum group (fig. 26A). Water intake in caloric restriction group was significantly lesser when compared to ad-libitum throughout the period of study (fig. 26B). Caloric restriction resulted in a significant decrease in weight gain when compared to ad-libitum group starting from the first week and lasted throughout the period of study (fig. 26C). Mean arterial pressure was similar in both the groups (fig. 27A). Fasting blood glucose levels were similar in both the groups (fig. 27B). However, caloric restriction resulted in ~50% decrease in fasting plasma insulin levels (fig. 27C) and improvement of insulin sensitivity (QUICKI) (fig. 27D).

Caloric restriction reduced triglyceride levels by ~40% (Table 4). Total cholesterol levels were similar in both the groups (Table 4). However, we could not statistically evaluate HDL cholesterol levels because the levels were above the detection limit in all except one of ad-libitum and five of caloric restriction group (Table 4). Caloric restriction significantly decreased body weight and the ratios of

fat, heart and spleen to body weight, compared to ad-libitum group (Table 4). However, ratio of kidney and liver to body weight were similar in both the groups (Table 4).

4.3.2 Effect of caloric restriction on oxidative stress:

4.3.2.1 Plasma and urinary 8-isoprostane:

Caloric restriction decreased plasma 8-isoprostane by ~20% (fig. 28A). In contrast, urinary 8-isoprostane levels were approximately three fold higher in caloric restriction group (fig. 28B).

4.3.2.2 Renal cortical protein carbonylation:

Caloric restriction did not alter the levels of protein carbonylation when compared with ad-libitum group (fig. 29). Since no bands were detected for derivatization controls in both the groups (fig. 29, upper panel, lanes 2 and 4), the entire strip of protein bands was used for quantification.

4.3.2.3 Renal cortical nitrosylation:

Caloric restriction did not reduce protein nitrotyrosine content in renal cortices when compared with ad-libitum group (fig. 30).

4.3.2.4 Plasma and renal cortical superoxide dismutase:

Caloric restriction did not affect the levels of superoxide dismutase in both the plasma (fig. 31A) and renal cortical homogenates (fig. 31B).

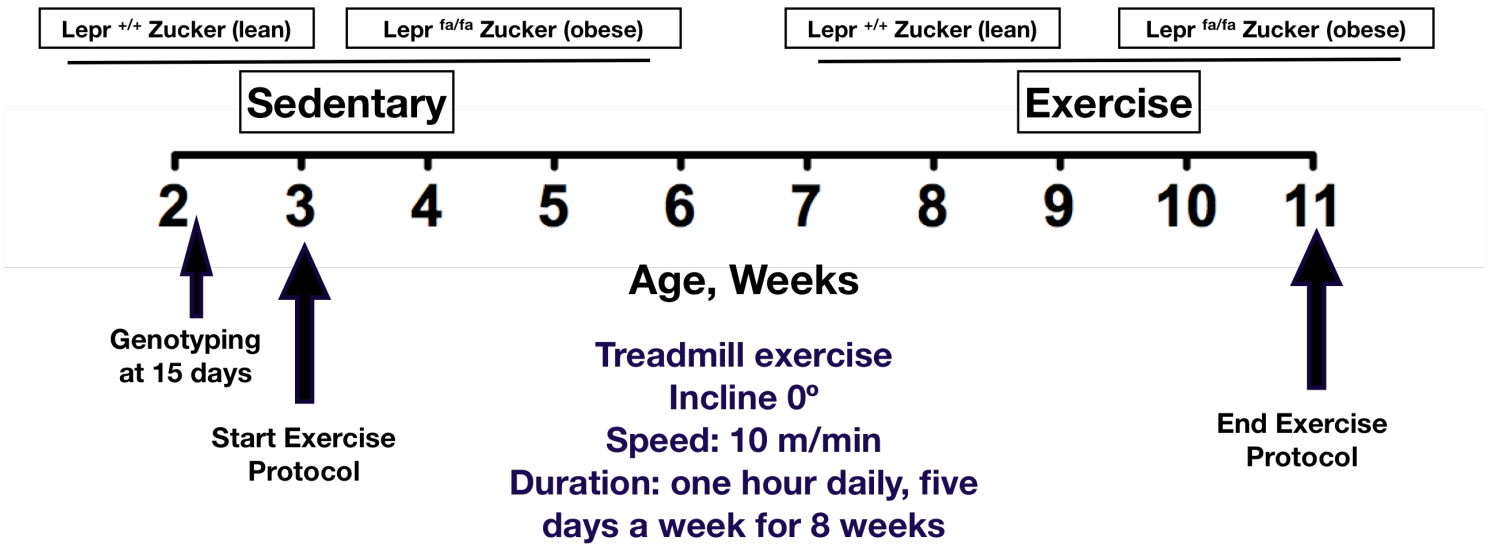
4.3.2.5 Renal cortical malondialdehyde:

Caloric restriction did not reduce renal cortical malondialdehyde levels when compared to ad-libitum group (fig. 32).

4.3.3 Effect of caloric restriction on natriuretic response to SKF38393:

Both caloric restriction and ad-libitum group displayed similar basal diuresis (fig. 33) and urinary sodium excretion (fig. 34). While SKF38393 significantly increased diuresis in both groups (fig. 33) it failed to increase urinary sodium excretion in both the groups (fig. 34).

Schematic 1: Exercise Protocol



Schematic 2:
Protocol to study sodium excretion in response to D1 receptor agonist (SKF38393)

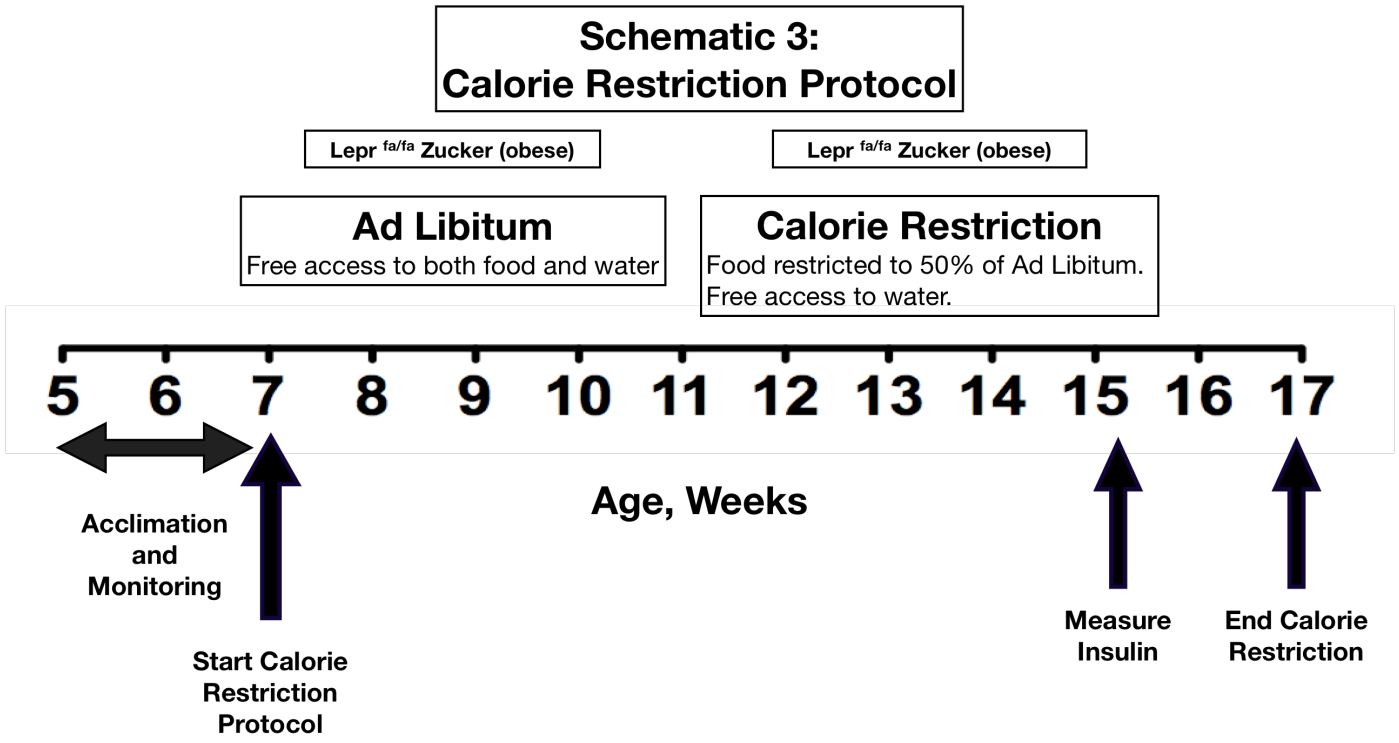

Schematic 3:
Calorie Restriction Protocol
Lepr fa/fa Zucker (obese)
Lepr fa/fa Zucker (obese)
Ad Libitum
Free access to both food and water
Calorie Restriction
Food restricted to 50% of Ad Libitum.
Free access to water.
5 6 7 8 9 10 11 12 13 14 15 16 17
Age, Weeks
Acclimation and Monitoring
Start Calorie Restriction Protocol
Measure Insulin
End Calorie Restriction

Table 2 Kidney weight and lipid profile in three week old $Lepr^{+/+}$ Zucker (lean) and $Lepr^{fa/fa}$ Zucker (obese) rats

	$Lepr^{+/+}$ (Lean)	$Lepr^{fa/fa}$ (Obese)
Body weight (grams)	76.83 ± 6.55	83.33 ± 4.09
Kidney:Body weight	0.0077 ± 0.0002	0.0071 ± 0.0006
Total Cholesterol (mg/dL)	120.5 ± 6.49	166.7 ± 10.5*
HDL cholesterol (mg/dL)	45.00 ± 3.0	78.4 ± 2.77*
Triglycerides (mg/dL)	64 ± 14.0	222 ± 38.91*

Data is expressed as Mean±SEM of n=5-6. **p<0.05 vs. $Lepr^{+/+}$ Zucker (lean) was considered significant using unpaired t-test.*

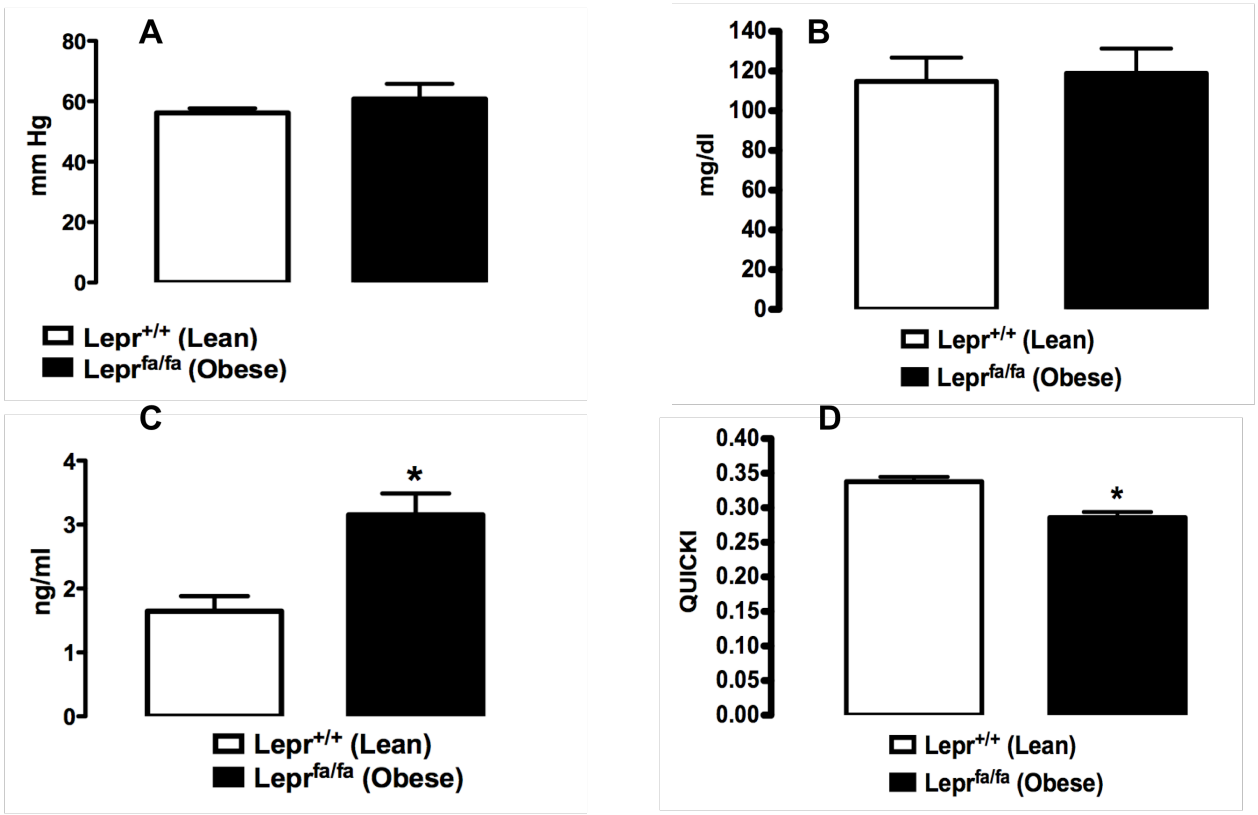


Fig. 1 (A) Mean arterial pressure (MAP), (B) fasting blood glucose and (C) fasting plasma insulin, (D) quantitative insulin check index (QUICKI) in three week old Lepr+/+ Zucker (lean) and Leprfa/fa Zucker (obese) rats: Data is expressed as Mean±SEM of n=5-6. *p<0.05 vs. Lepr+/+ Zucker (lean) was considered significant using unpaired t-test.

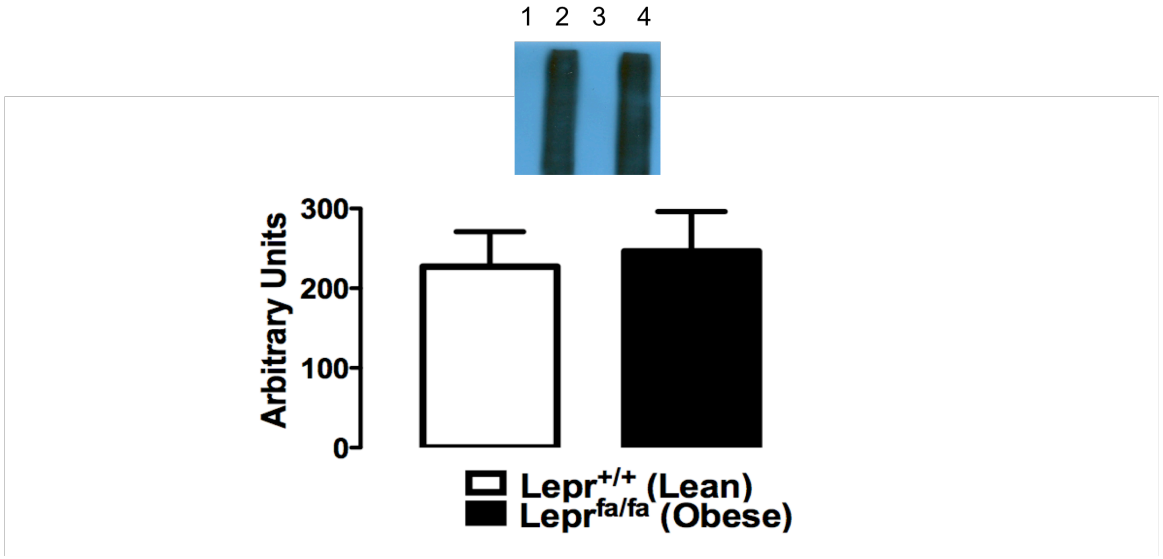


Fig. 2 Protein carbonylation in renal proximal tubular homogenate from three week old Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats: *Upper panel:* Representative blot showing bands detected by antibody specific for 2, 4 dinitrophenylhydrazone (DNP)-hydrazone-derivatives of carbonylated proteins for lean (lane 2) and obese (lane 4) rats. No bands were detected for derivatization controls in both lean (lane 1) and obese (lane 3) rats. *Lower panel:* Data is expressed as Mean±SEM of n=5-6.

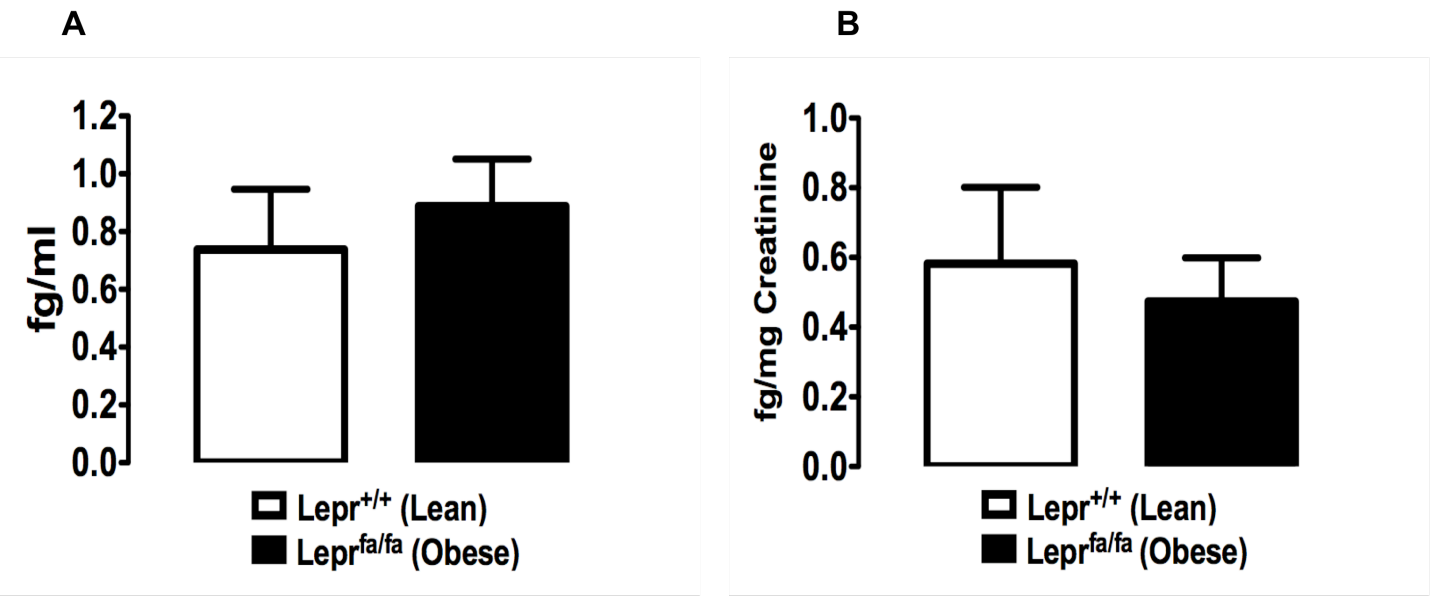


Fig. 3 (A) Plasma 8-isoprostane and (B) Urinary 8-isoprostane in three week old Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats: 8-Isoprostane levels were measured by a competitive enzyme immunosorbent assay (EIA). Urinary 8-isoprostane levels were normalized using urinary creatinine levels. Data is expressed as Mean±SEM of n=5-6.

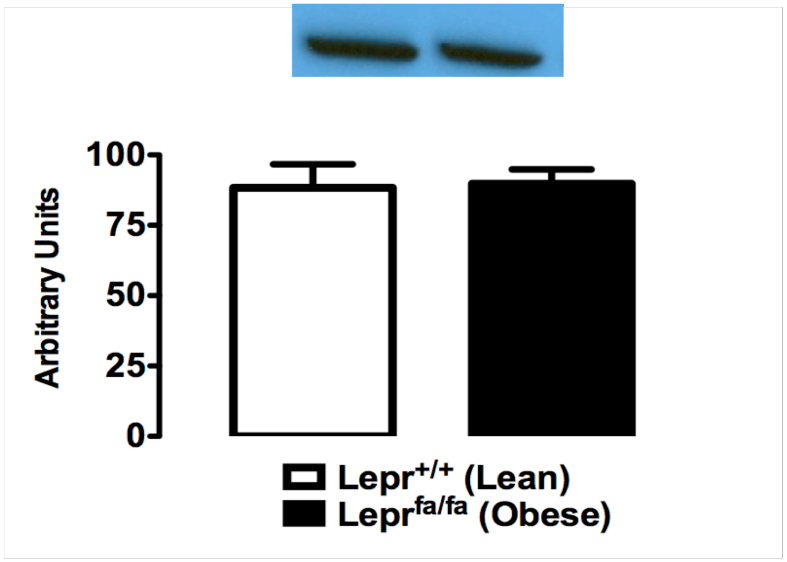


Fig. 4 Protein nitrosylation in renal proximal tubular homogenate from three week old Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats: *Upper panel:* Representative blot showing bands detected by HRP conjugated anti-nitrotyrosine (clone 1A6) IgG. *Lower panel:* Data is expressed as Mean±SEM of n=5-6.

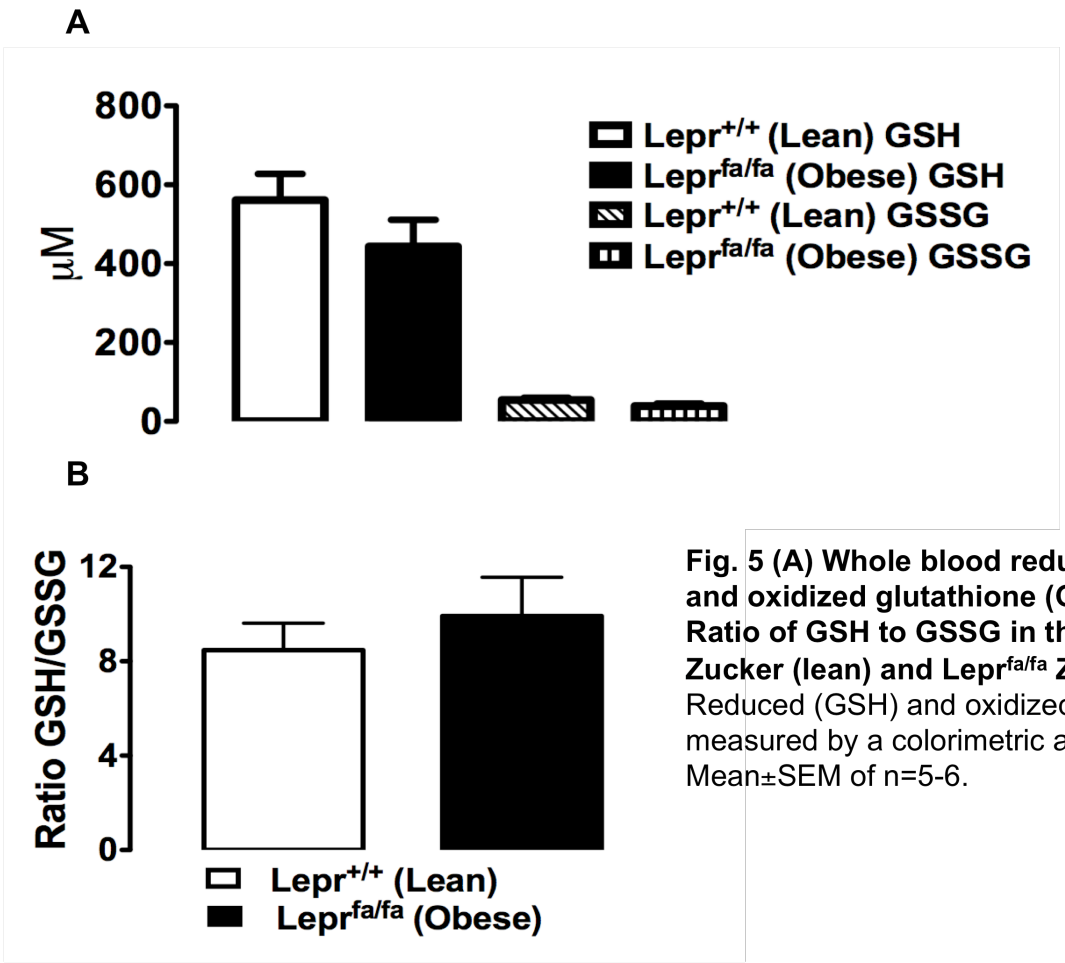


Fig. 5 (A) Whole blood reduced glutathione (GSH) and oxidized glutathione (GSSG) levels and (B) Ratio of GSH to GSSG in three week old Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats: Reduced (GSH) and oxidized (GSSG) glutathione were measured by a colorimetric assay. Data is expressed as Mean±SEM of n=5-6.

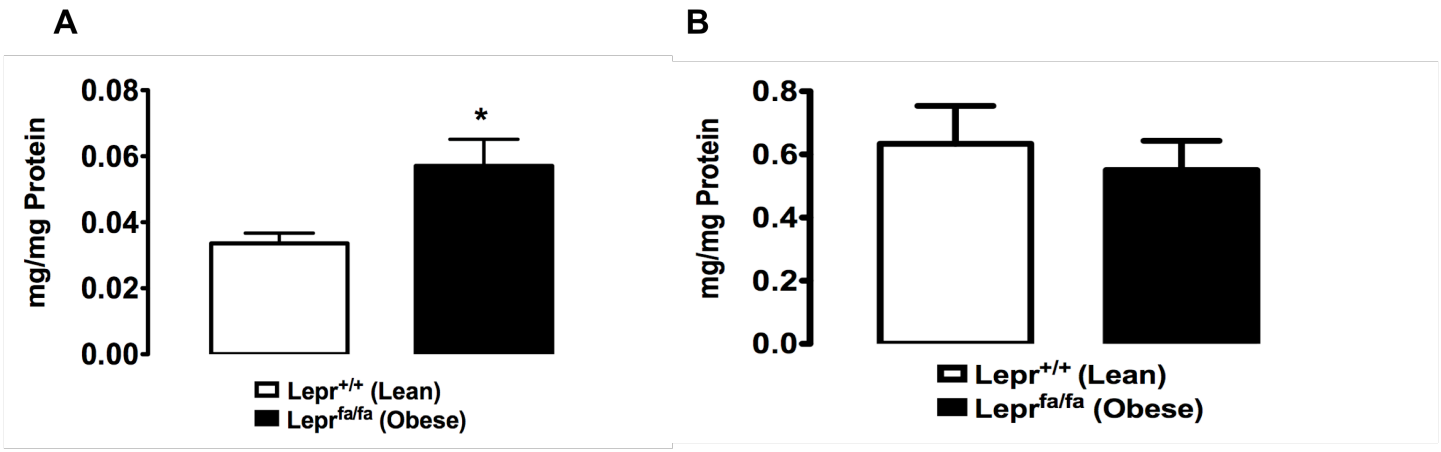


Fig. 6 Hemeoxygenase-1 (HO-1) levels in (A) Plasma and (B) renal proximal tubular homogenate from three week old Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats: Hemeoxygenase-1 (HO-1) levels were measured by a sandwich enzyme linked immunosorbent assay (ELISA). Data is expressed as Mean±SEM of n=5-6. **p<0.05 vs. Lepr$^{+/+}$ Zucker (lean) was considered significant* using *unpaired t-test.*

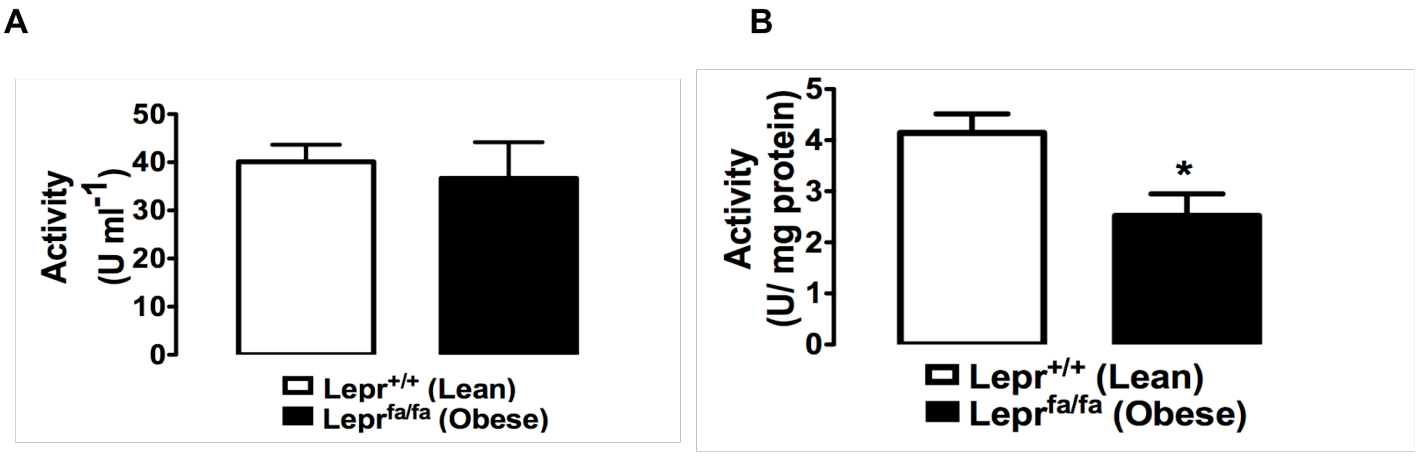


Fig. 7 Superoxide dismutase (SOD) activity in (A) Plasma and (B) renal proximal tubular homogenate from three week old Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats: SOD activity was measured using a colorimetric assay. Data is expressed as Mean±SEM of n=5-6. **p<0.05 vs. Lepr$^{+/+}$ Zucker (lean) was considered significant* using *unpaired t-test.*

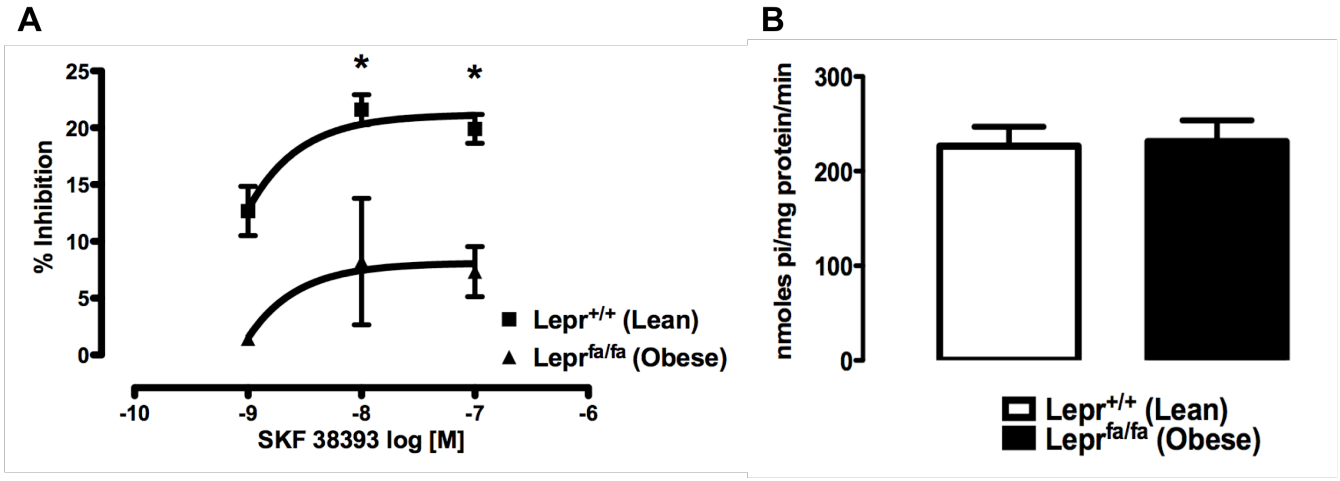


Fig. 8 (A) SKF38393 mediated inhibition of Na^+/K^+ ATPase activity, expressed as percentage of basal Na^+/K^+ ATPase activity (B) Basal Na^+/K^+ ATPase activity, in proximal tubular preparation from three week old $Lepr^{+/+}$ Zucker (lean) and $Lepr^{fa/fa}$ Zucker (obese) rats: Data is expressed as Mean±SEM of n=4-5. **p<0.05 vs. $Lepr^{+/+}$ Zucker (lean) was considered significant using unpaired t-test.*

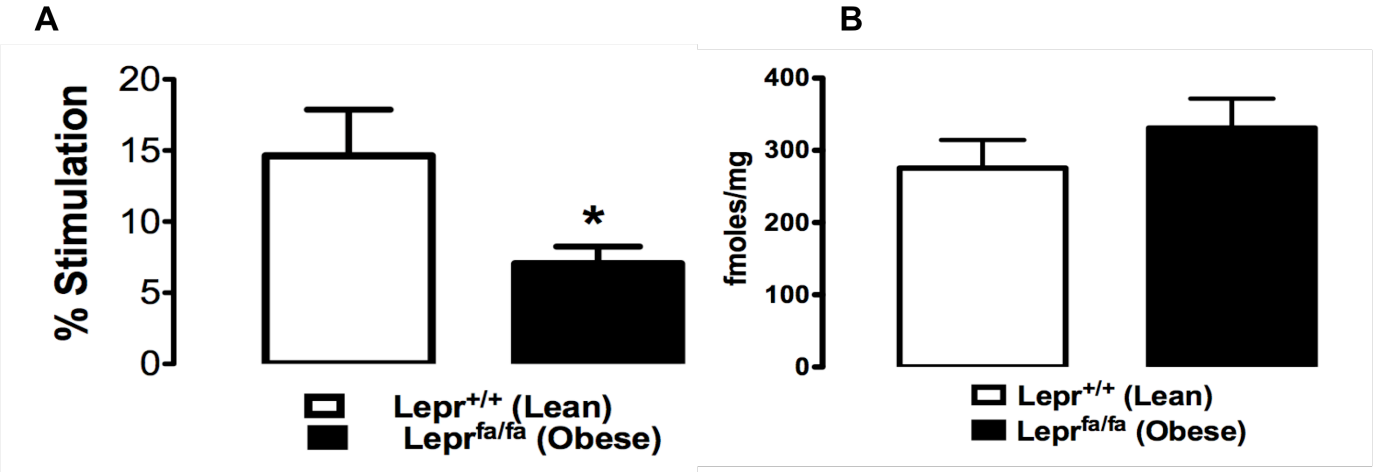


Fig. 9 (A) SKF38393 mediated stimulation of [^{35}S]GTPγS binding, expressed as percentage over basal [^{35}S]GTPγS binding (B) Basal [^{35}S]GTPγS binding, in proximal tubular membranes from three week old Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats: Data is expressed as Mean±SEM of n=4-5. **p<0.05 vs. Lepr$^{+/+}$ Zucker (lean) was considered significant* using *unpaired t-test.*

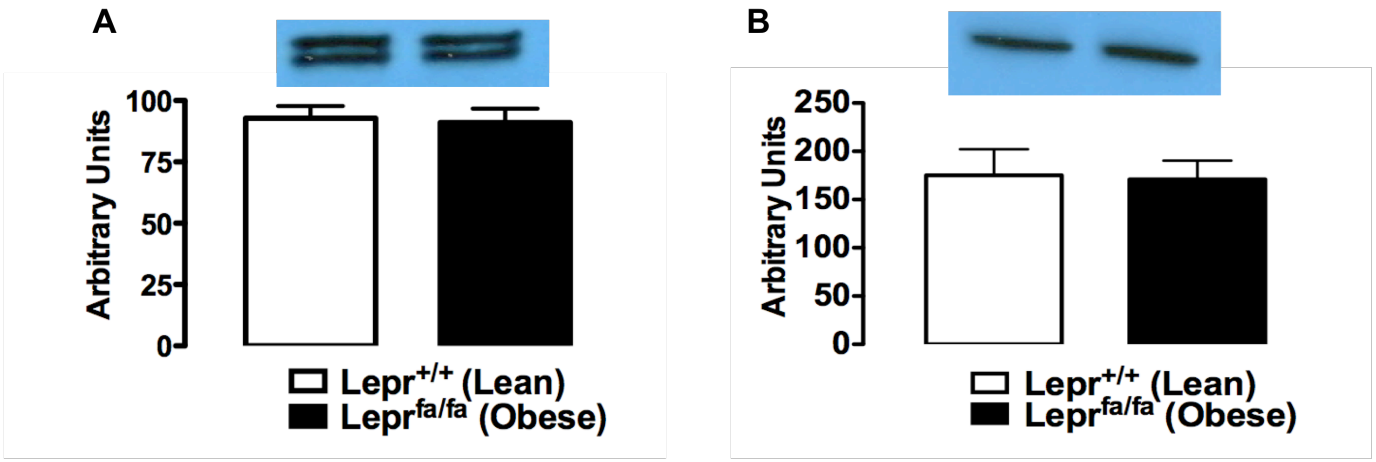


Fig. 10 (A) $G_{s\alpha}$ and (B) $G_{q/11\alpha}$ levels, in proximal tubular homogenates from three week old $Lepr^{+/+}$ Zucker (lean) and $Lepr^{fa/fa}$ Zucker (obese) rats: *Upper panels:* Representative blots showing bands detected by anti-$G_{s\alpha}$ and and anti- $G_{q/11\alpha}$, respectively. *Lower panels:* Data is expressed as Mean±SEM of n=4-5.

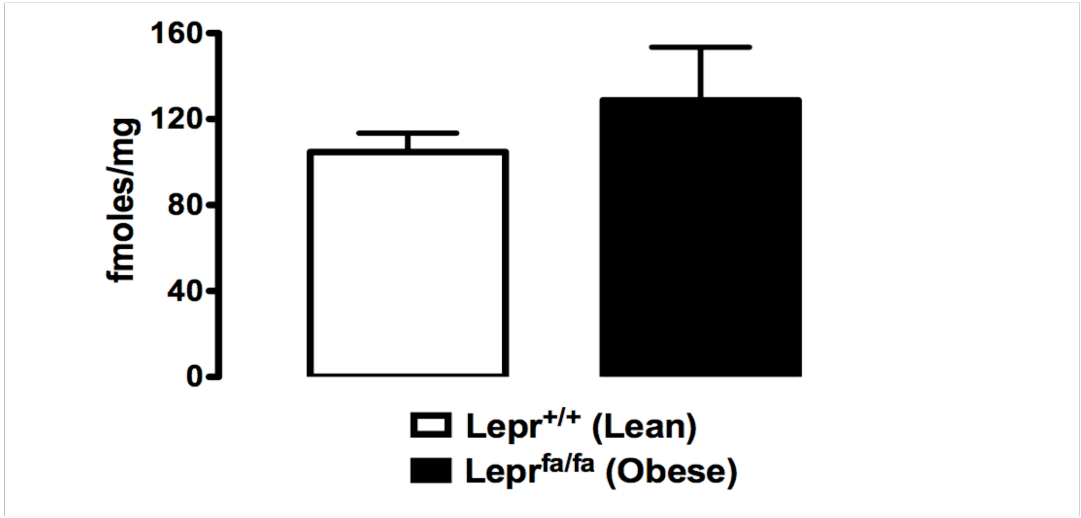


Fig. 11 Dopamine D1 receptor radioligand ([^{3}H]SCH23390) binding in proximal tubular membranes from three week old Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats: Bars represent specific binding using 20nM [^{3}H]SCH23390. Data is expressed as Mean±SEM of n=4-5.

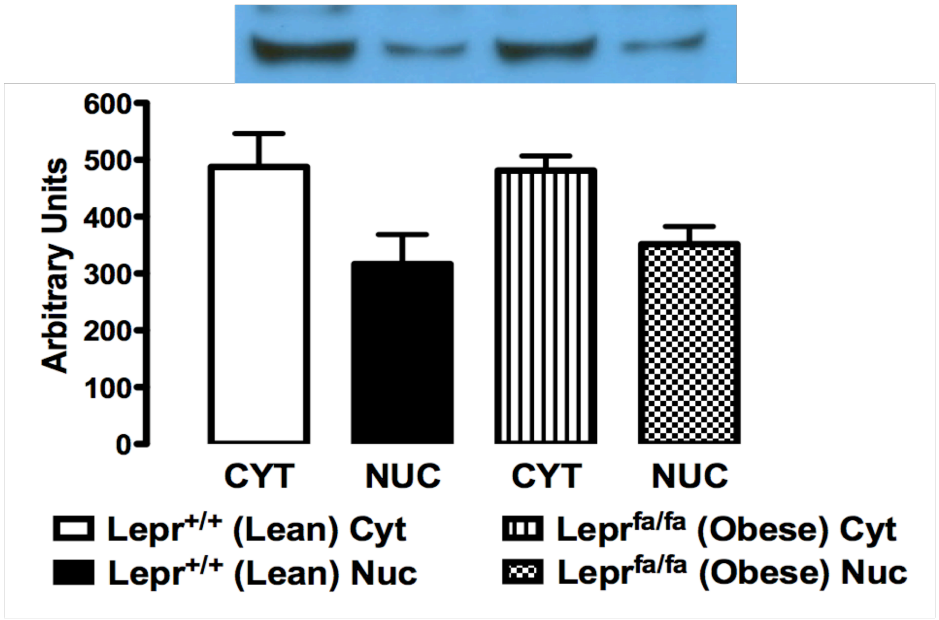


Fig. 12 Nuclear factor κ B (NFκB) levels in renal proximal tubular nuclear and cytosolic fractions from three week old Lepr$^{+/+}$ Zucker (lean) and Lepr$^{fa/fa}$ Zucker (obese) rats: *Upper panel:* Representative blot showing bands detected by anti-NFκB IgG. *Lower panel:* Bars represent mean band intensity per unit area. Data is expressed as Mean±SEM of n=5-6.

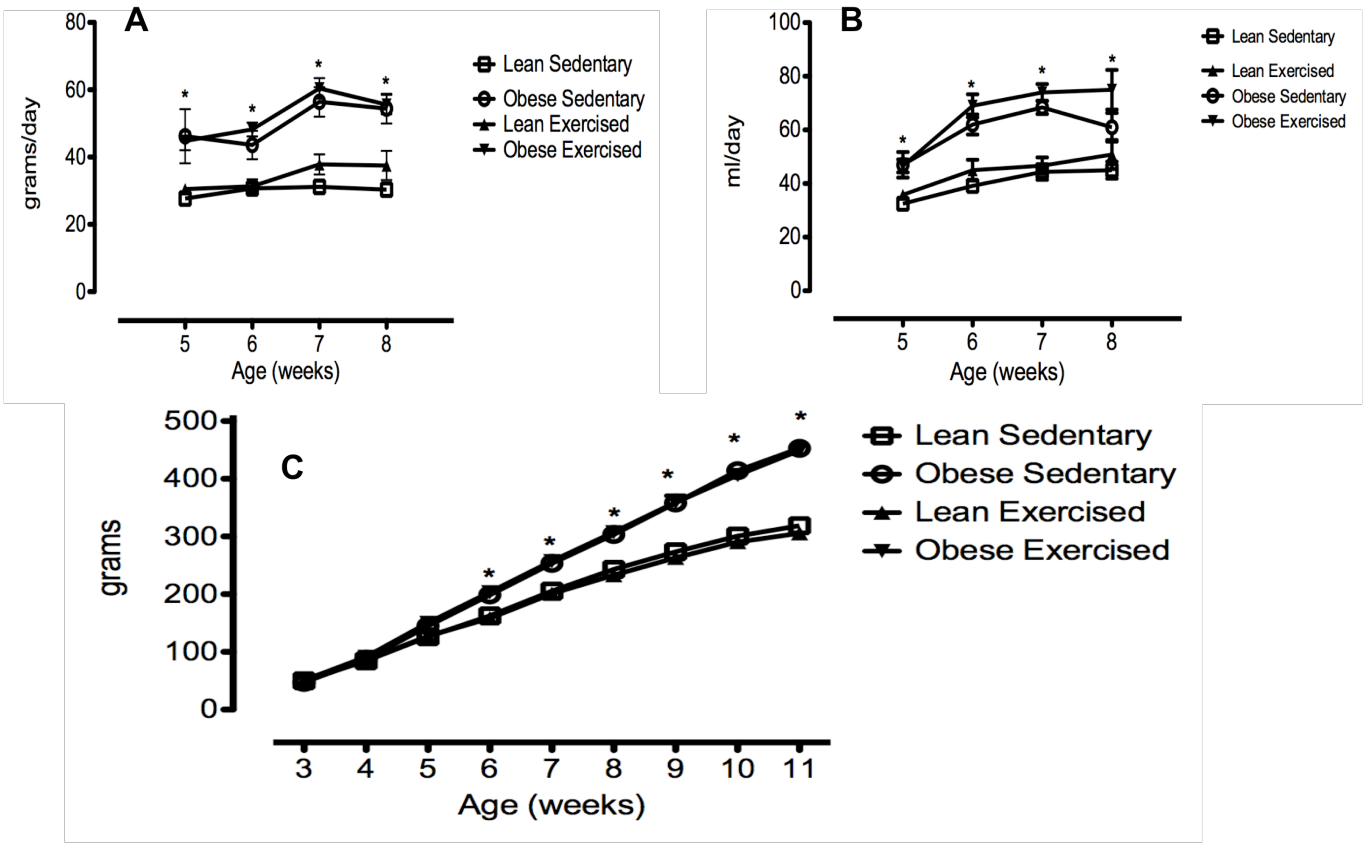


Fig. 13 Effect of exercise on (A) food intake (B) water intake (C) body weight: Data is expressed as Mean±SEM of n=8-10. **p<0.05 vs. lean rats was considered significant* using ANOVA followed by Newman-Keuls multiple test.

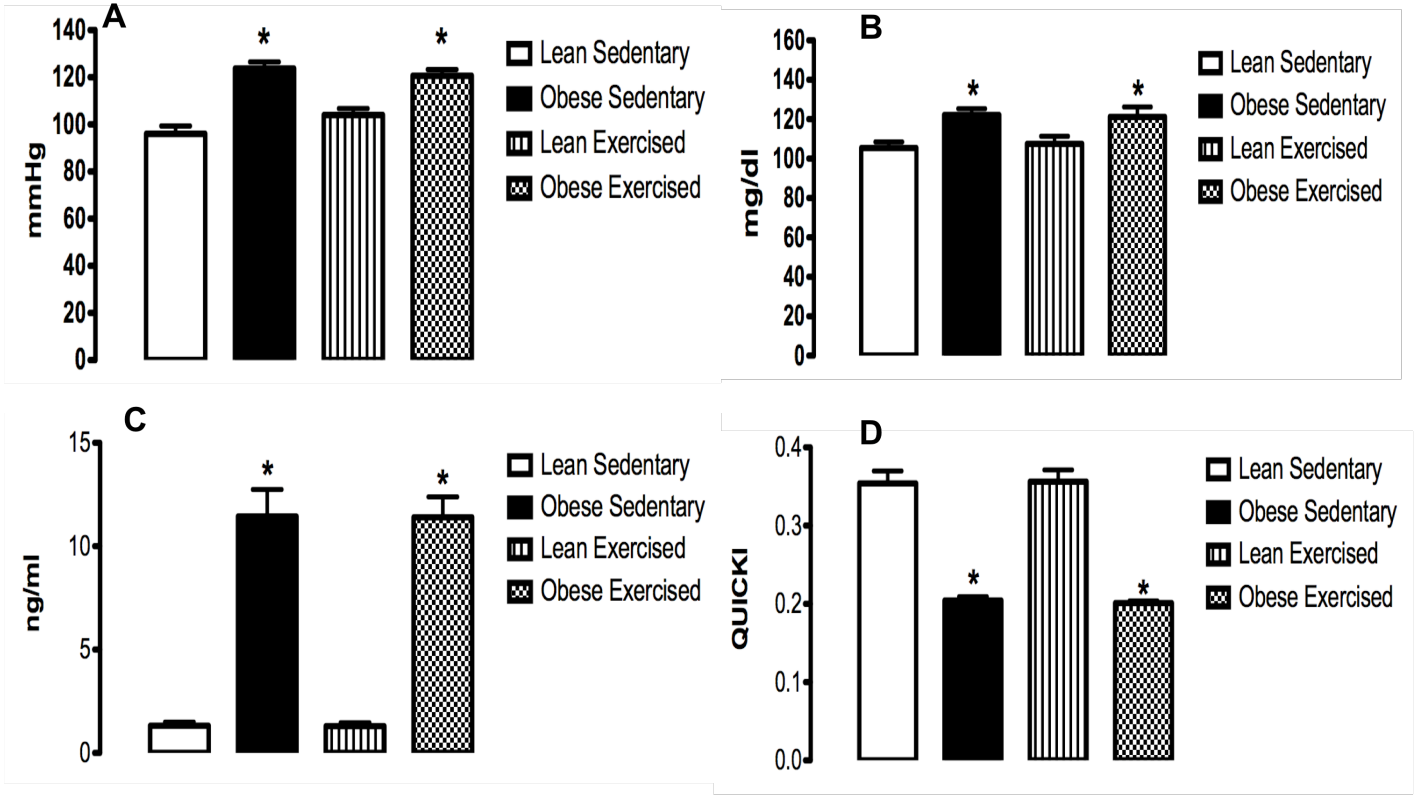


Fig. 14 Effect of exercise on (A) mean arterial pressure (MAP) (B) fasting blood glucose (C) fasting plasma insulin (D) quantitative insulin check index (QUICKI): Data is expressed as Mean±SEM of n=8-10. **p<0.05 vs. lean rats was considered significant* using ANOVA followed by Newman-Keuls multiple test.

Table 3 Effect of exercise on lipid profile and organ weights

	Lean Sedentary	Obese Sedentary	Lean Exercised	Obese Exercised
Triglycerides (mg/dL)	63.00±3.81	359.8±39.77*	61.11±1.77	276.1±37.66*#
Total Cholesterol (mg/dL)	BDL	124.0±5.77	BDL	119.7±5.77
HDL cholesterol (mg/dL)	31.18±1.53	58.00±5.65*	29.73±2.49	47.55±2.92*#
Body weight (grams)	296±4.32	440±15.61*	295.8±6.66	459.2±8.65*
Fat:Body Weight	0.02654± 0.002398	0.09932 ±0.0063364*	0.02530 ±0.001100	0.1139 ±0.002263*#
Kidney:Body weight	0.004715±0.00010	0.003970±0.00015*	0.004863±0.00017	0.003995±0.00020*
Heart:body weight	0.003332±0.00008	0.002712±0.00005*	0.003424±0.00007	0.002741±0.00006*
Spleen:Body weight	0.001949±0.00015	0.001466±0.00009*	0.002122±0.00018	0.001447±0.00006*
Brain:Body weight	0.006704±0.00034	0.004388±0.00015*	0.007046±0.00022	0.004279±0.00019*
Liver:Body weight	0.03296±0.0011	0.03390±0.002	0.03036±0.0014	0.03588±0.0019

Data is expressed as Mean±SEM of n=8-10. **p<0.05 vs. lean rats and #p<0.05 vs. obese sedentary was considered significant* using ANOVA followed by Newman-Keuls multiple test and unpaired t-test for total cholesterol. BDL-below detection limit.

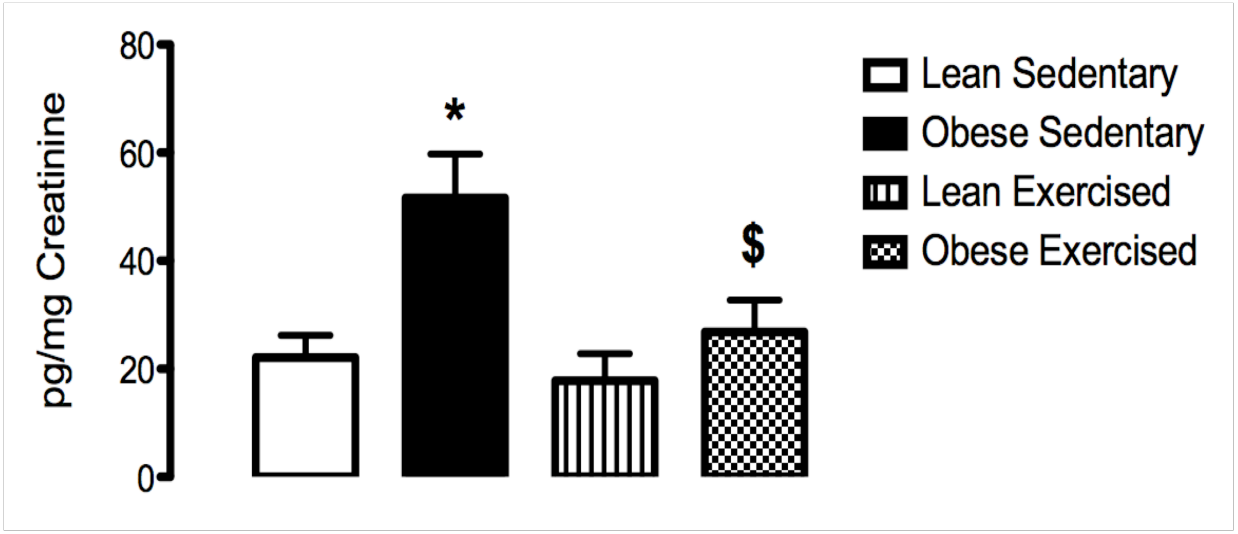


Fig. 15 Effect of exercise on urinary 8-isoprostane: Bars represent urinary 8-Isoprostane levels measured by a competitive enzyme immunosorbent assay (EIA) and normalized by urinary creatinine levels. Data is expressed as Mean±SEM of n=8-10. **p<0.05 vs. lean rats and $p<0.05 vs. obese sedentary rats was considered significant* using ANOVA followed by Newman-Keuls multiple test.

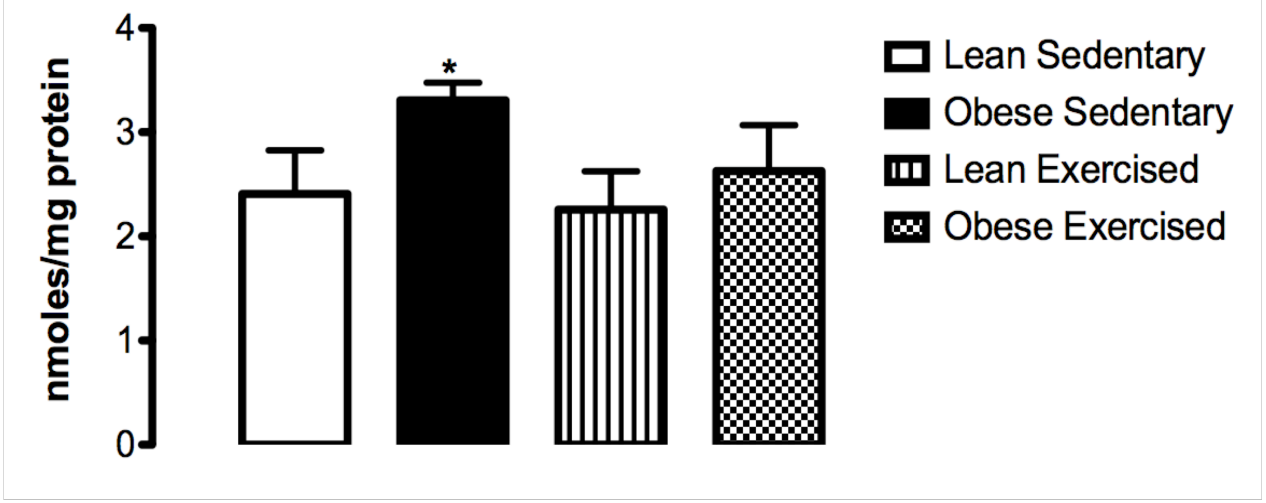


Fig. 16 Effect of exercise on proximal tubular malondialdehyde (MDA): MDA levels were measured as thiobarbituric acid reactive substances (TBARS) in proximal tubular homogenates by a colorimetric assay. Data is expressed as Mean±SEM of n=8-10. **p<0.05 vs. lean rats was considered significant* using ANOVA followed by Newman-Keuls multiple test.

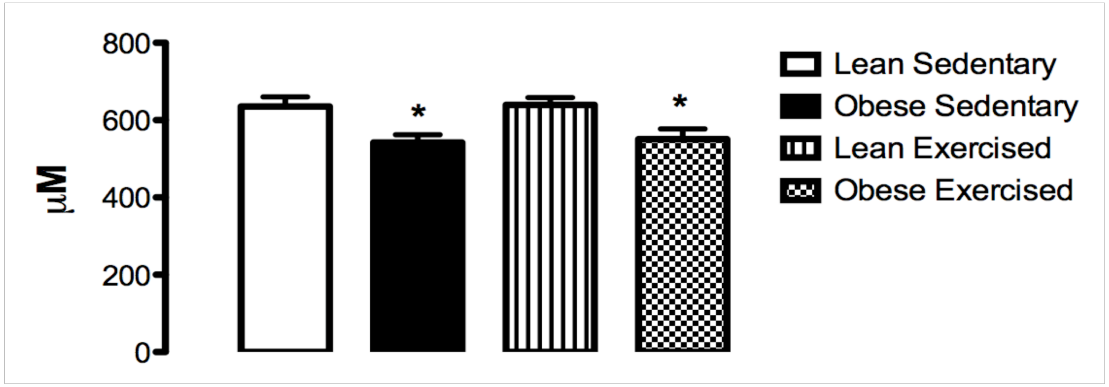


Fig. 17 Effect of exercise on blood total glutathione levels: Total glutathione levels were measured by a colorimetric assay. Data is expressed as Mean±SEM of n=8-10. **p<0.05 vs. lean rats was considered significant* using ANOVA followed by Newman-Keuls multiple test.

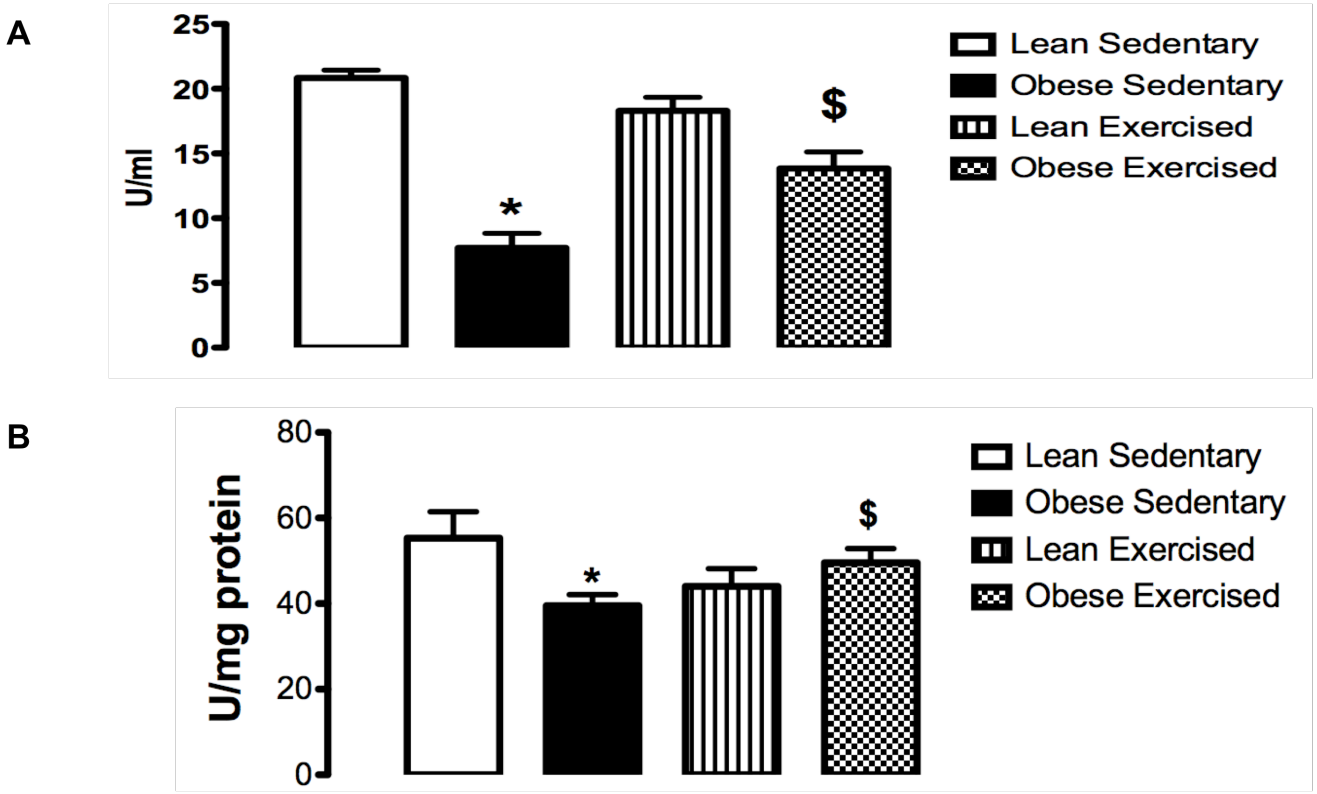


Fig. 18 Effect of exercise on (A) plasma superoxide dismutase (SOD) activity (B) proximal tubular homogenate SOD activity: SOD activity was measured using a colorimetric assay. Data is expressed as Mean±SEM of n=8-10. **p<0.05 vs. lean rats and $p<0.05 vs. obese sedentary rats was considered significant* using ANOVA followed by Newman-Keuls multiple test.

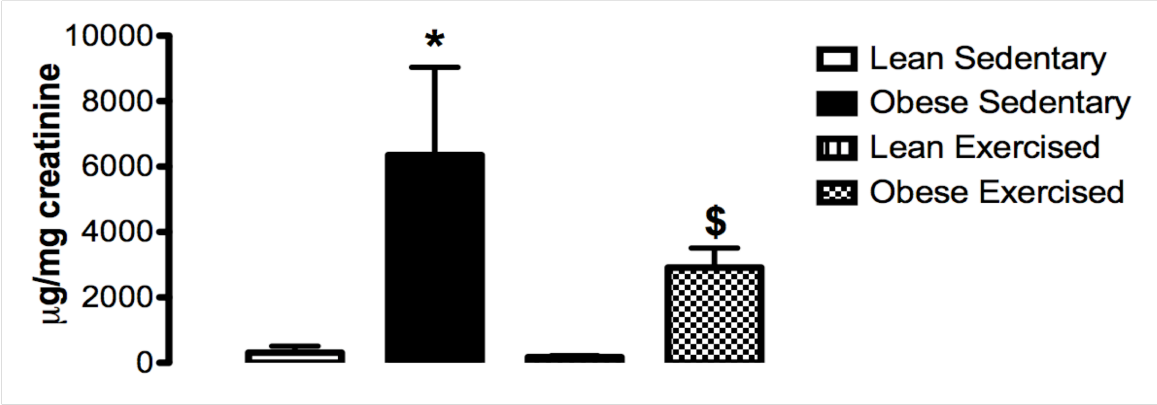


Fig. 19 Effect of exercise on urinary albumin levels: Urinary albumin was measured by an enzyme immuno-assay (EIA). Data is expressed as Mean±SEM of n=8-10. **p<0.05 vs. lean rats and $p<0.05 vs. obese sedentary rats was considered significant* using ANOVA followed by Newman-Keuls multiple test.

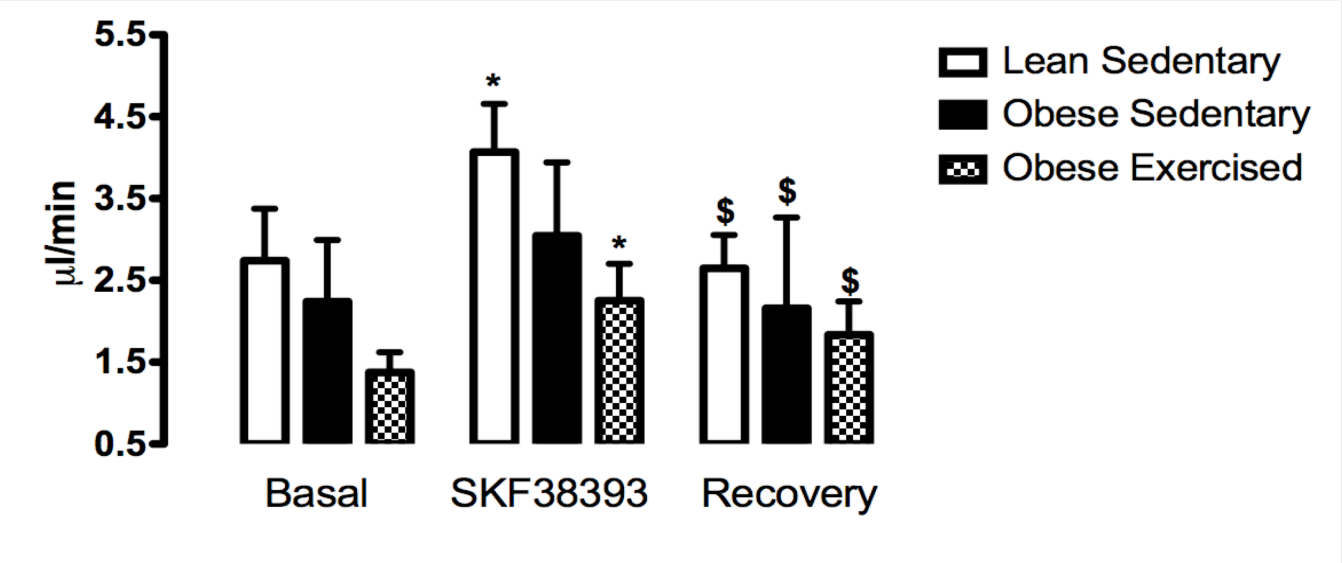


Fig. 20 Effect of exercise on urine flow: Bars represent urine flow (µl/min) calculated using one 30 min collection during SKF38393 phase and an average of two 30 min collections in basal and recovery phase. Data is expressed as Mean±SEM of n=5-6. **p<0.05 vs basal and $ p<0.05 vs SKF38393 was considered significant* using ANOVA followed by Newman-Keuls multiple test.

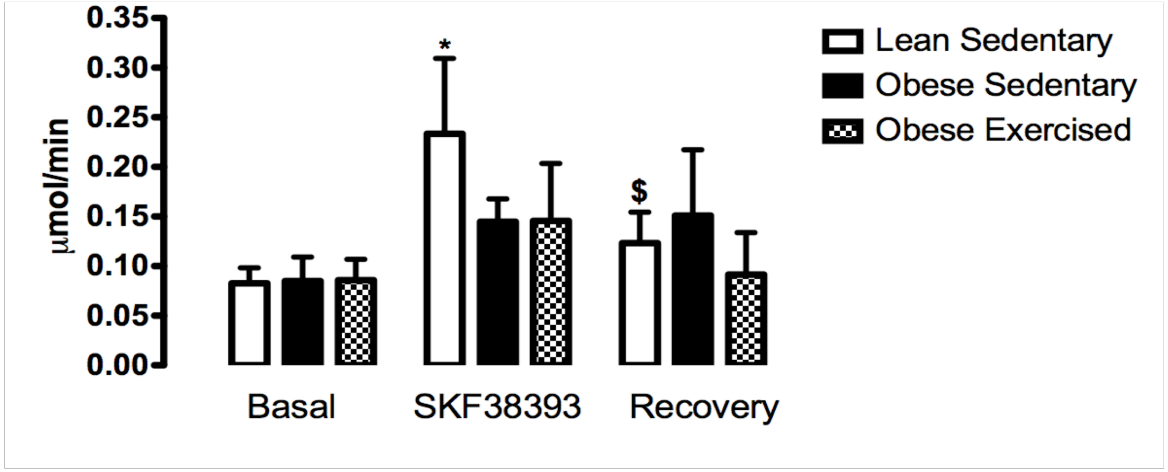


Fig. 21 Effect of exercise on urinary sodium excretion ($U_{Na}V$): Bars represent $U_{Na}V$ (µmol/min) calculated using one 30 min collection during SKF38393 phase and an average of two 30 min collections in basal and recovery phase. Data is expressed as Mean±SEM of n=5-6. **p<0.05 vs basal and $ p<0.05 vs SKF38393 was considered significant* using ANOVA followed by Newman-Keuls multiple test.

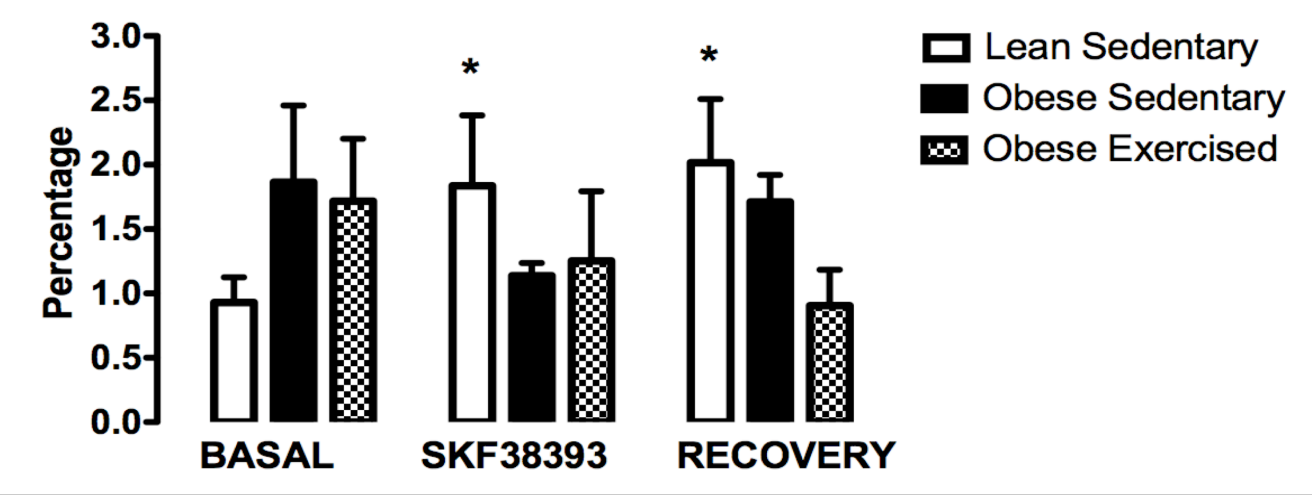


Fig. 22 Effect of exercise on fractional excretion of sodium (FE_{Na}): Bars represent FE_{Na} calculated using one 30 min collection during SKF38393 phase and an average of two 30 min collections in basal and recovery phase. Data is expressed as Mean±SEM of n=5-6. **p<0.05 vs basal and $ p<0.05 vs SKF38393 was considered significant* using ANOVA followed by Newman-Keuls multiple test.

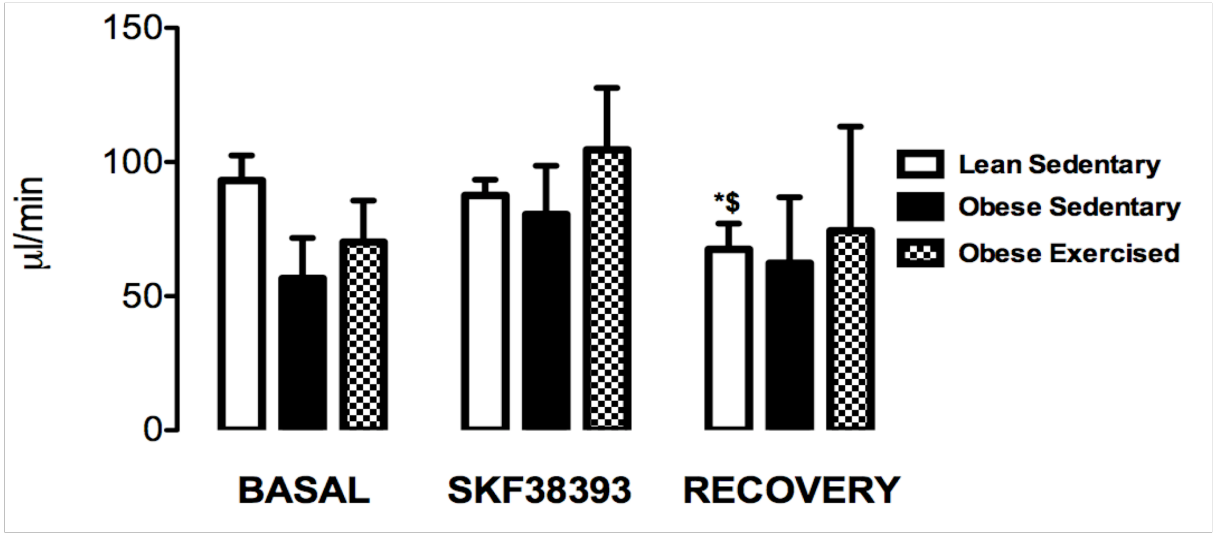


Fig. 23 Effect of exercise on glomerular filtration rate (GFR): Bars represent GFR (µl/min) calculated using one 30 min collection during SKF38393 phase and an average of two 30 min collections in basal and recovery phase. Data is expressed as Mean±SEM of n=5-6. **p<0.05 vs basal and $ p<0.05 vs SKF38393 was considered significant* using ANOVA followed by Newman-Keuls multiple test.

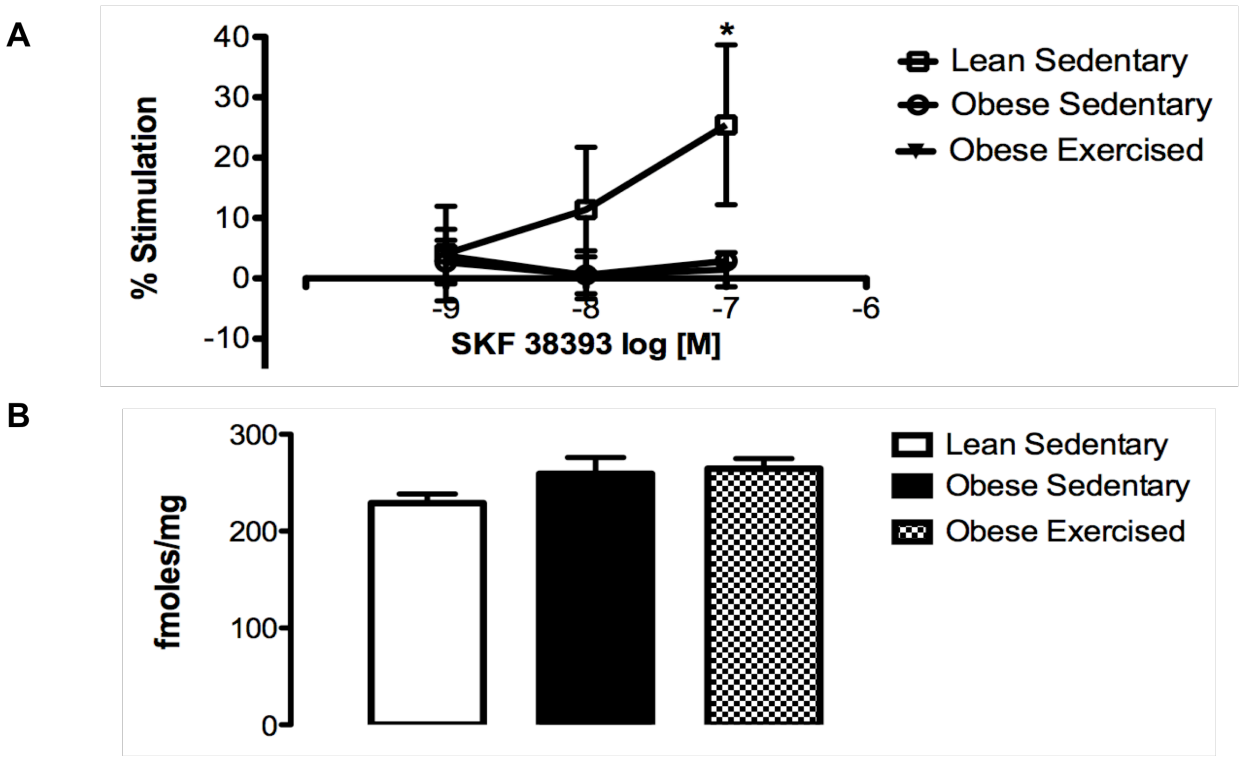


Fig. 24 Effect of exercise on (A) Stimulation of membrane [^{35}S]GTPγS binding in response to *SKF38393,* expressed as percentage over basal [^{35}S]GTPγS binding *(B)* basal [^{35}S]GTPγS binding*:* Data is expressed as Mean±SEM of n=5. **p<0.05 vs. obese rats was considered significant* using ANOVA followed by Newman-Keuls multiple test.

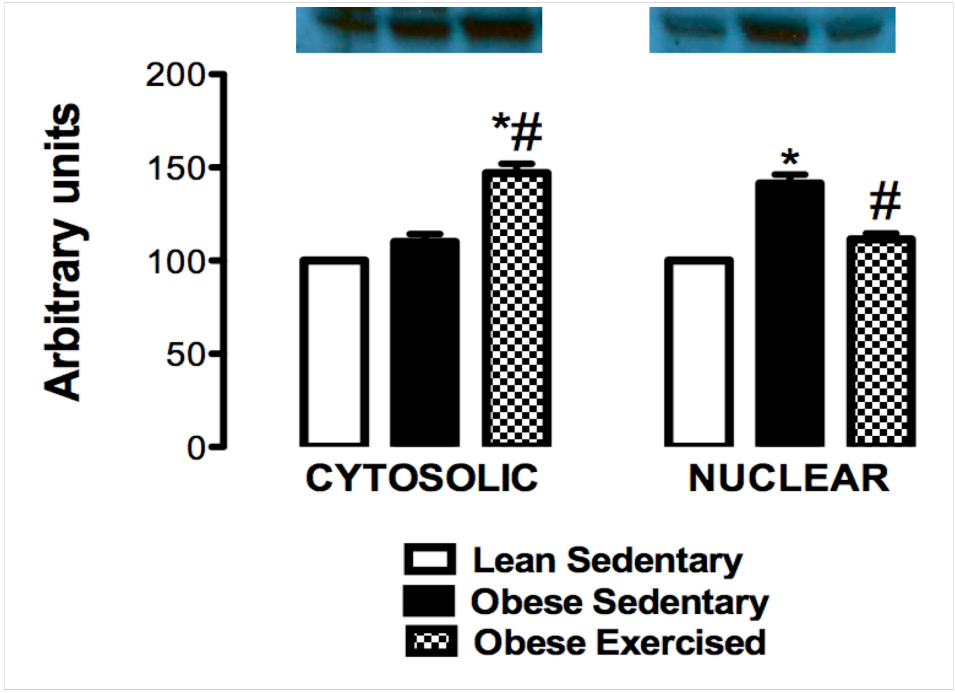


Fig. 25 Effect of exercise on nuclear translocation of NFκB: *Upper panel:* Representative blot showing bands detected by anti-NFκB IgG. *Lower panel:* Bars represent cytosolic and nuclear NFκB levels as a percentage of cytosolic and nuclear NFκB levels, respectively, in lean rats. Data is expressed as Mean±SEM of n=4. **p<0.05 vs. lean and #p<0.05 vs. obese sedentary was considered significant* using ANOVA followed by Newman-Keuls multiple test.

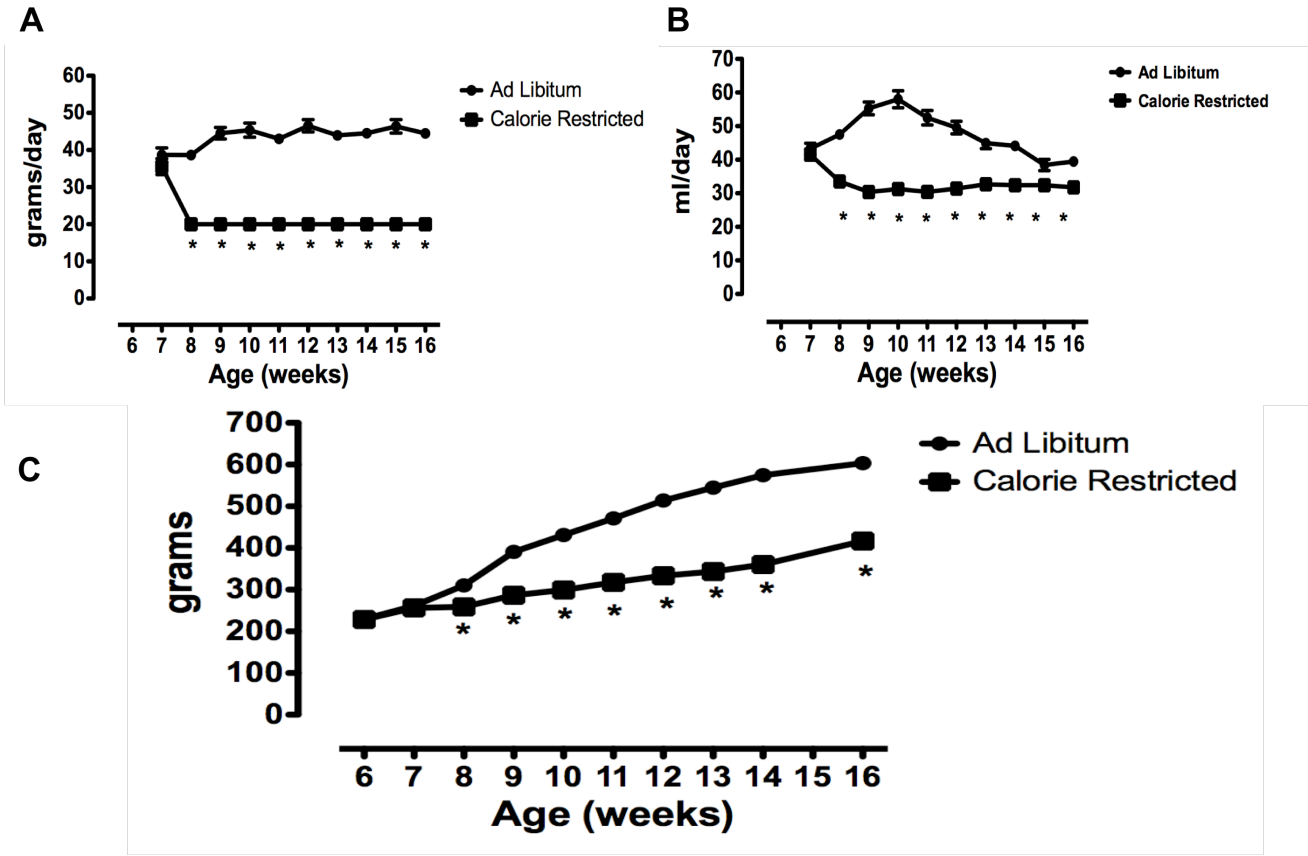


Fig. 26 Effect of caloric restriction on (A) food intake (B) water intake (C) body weight: Data is expressed as Mean±SEM of n=10-11. **p<0.05 vs. ad-libitum was considered significant* using ANOVA followed by Newman-Keuls multiple test.

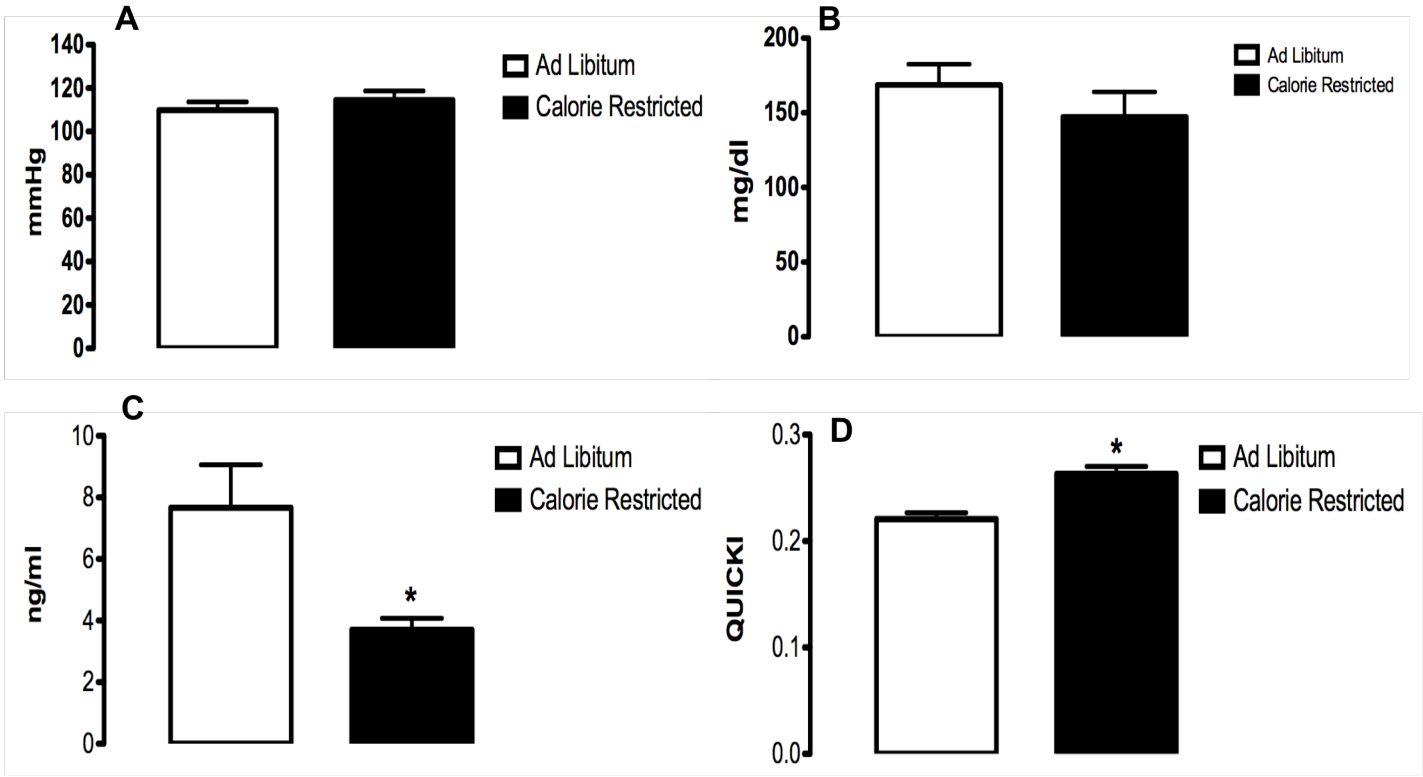


Fig. 27 Effect of caloric restriction on (A) mean arterial pressure (MAP) (B) fasting blood glucose (C) fasting plasma insulin (D) quantitative insulin check index (QUICKI): Data is expressed as Mean±SEM of n=10-11. **p<0.05 vs. ad-libitum was considered significant* using *unpaired t-test.*

Table 4 Effect of caloric restriction on lipid profile and organ weights

	Ad-libitum	Caloric Restricted
Triglycerides (mg/dL)	358 ± 28.1	215.7 ± 36.25*
Total Cholesterol (mg/dL)	171.8 ± 23.67	147.7 ± 10.02
HDL cholesterol (mg/dL) n=1 for AL,n=5 for CR	89 ± 0.00	70.4 ± 4.83
Body weight (grams)	603.6 ± 8.22	416.7 ± 6.65*
Fat:Body Weight	0.1075 ± 0.0078	0.0969 ± 0.0015*
Heart:body weight	0.001964 ± 0.00006	0.001393 ± 0.00005*
Spleen:Body weight	0.001116 ± 0.0001	0.0007527 ± 0.00002*
Kidney:Body weight	0.003120 ± 0.0002	0.002702 ±0.000007
Liver:Body weight	0.02900 ± 0.0009	0.02757 ± 0.0012

Data is expressed as Mean±SEM of n=8-10. **p<0.05 vs. ad-libitum was considered significant* using unpaired t-test.

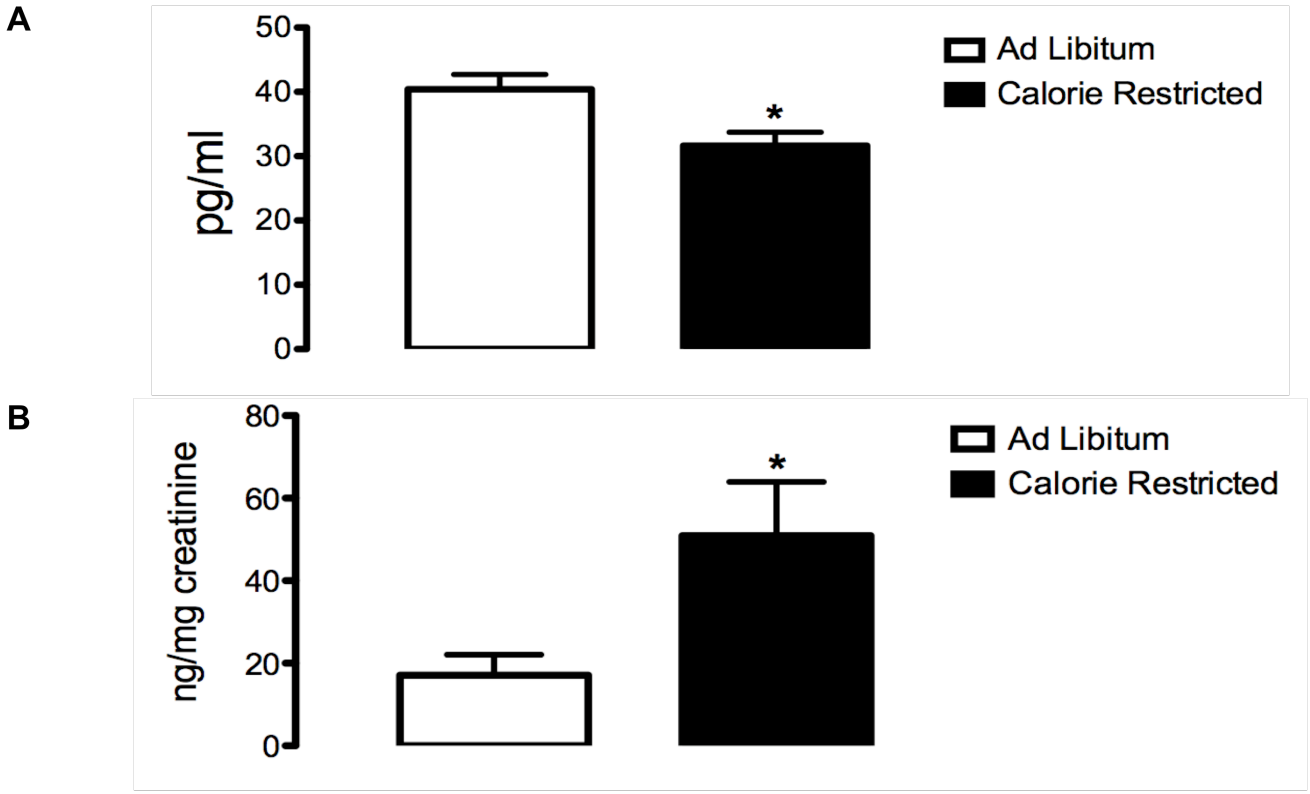


Fig. 28 Effect of caloric restriction on (A) plasma 8-isoprostane (B) urinary 8-isoprostane: 8-Isoprostane levels were measured by a competitive enzyme immunosorbent assay (EIA). Urinary 8-Isoprostane levels were normalized using urinary creatinine levels. Data is expressed as Mean±SEM of n=10-11. **p<0.05 vs. ad-libitum was considered significant* using *unpaired t-test.*

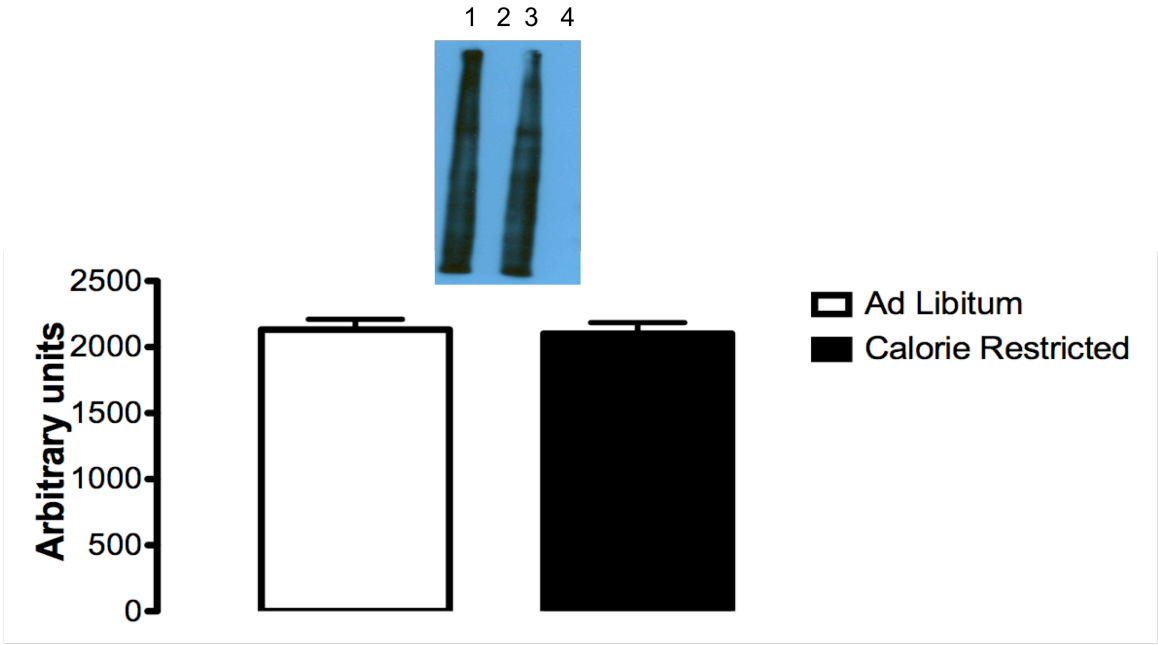


Fig. 29 Effect of caloric restriction on renal cortical protein carbonylation: *Upper panel:* Representative blot showing bands detected by antibody specific for 2, 4 dinitrophenylhydrazone (DNP)-hydrazone-derivatives of carbonylated proteins for ad-libitum (lane 1) and calorie restricted (lane 3) rats. No bands were detected for derivatization controls in both ad-libitum (lane 2) and calorie restricted (lane 4) rats. *Lower panel:* Data is expressed as Mean±SEM of n=10-11.

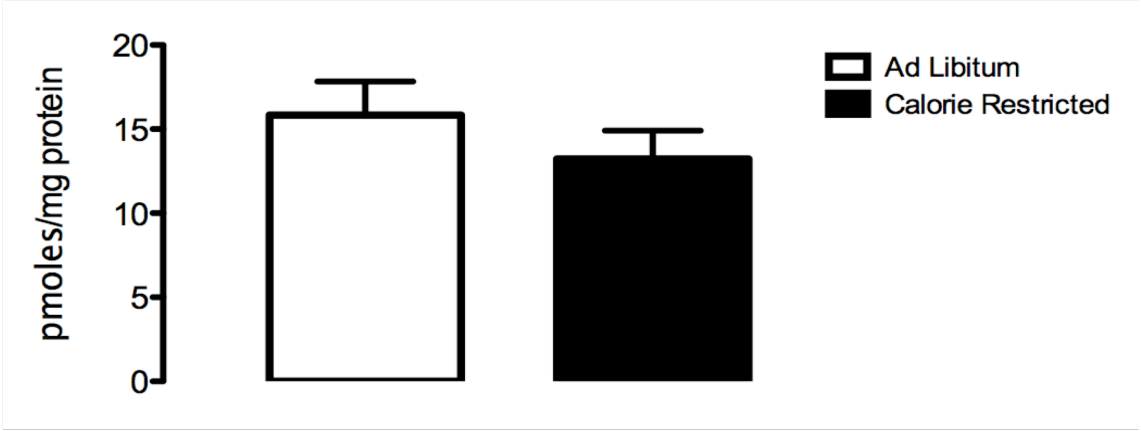


Fig. 30 Effect of caloric restriction on renal cortical protein nitrosylation: Renal cortical protein nitrosylation was measured by a competitive enzyme linked immunosorbent assay (ELISA). Data is expressed as Mean±SEM of n=10-11.

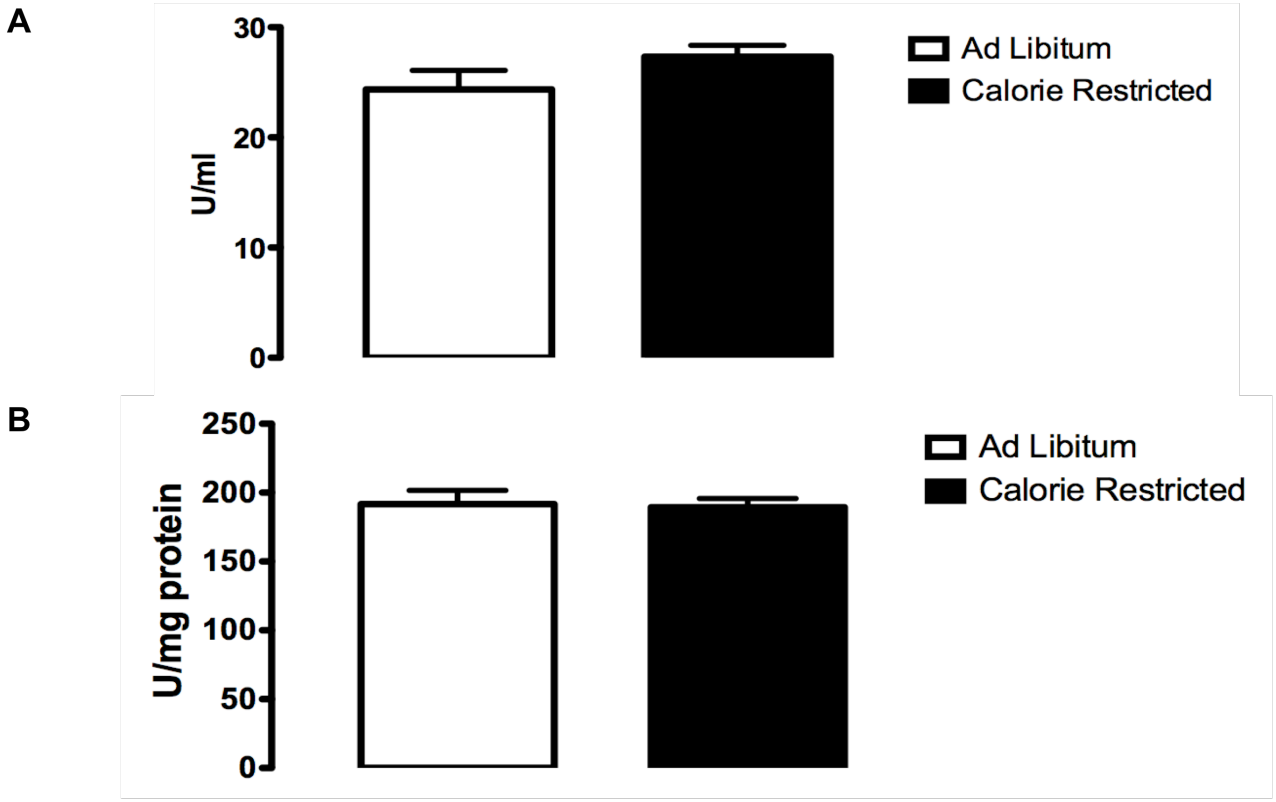


Fig. 31 Effect of caloric restriction on (A) plasma superoxide dismutase (SOD) activity (B) renal cortical homogenate SOD activity: SOD activity was measured using a colorimetric assay. Data is expressed as Mean±SEM of n=10-11.

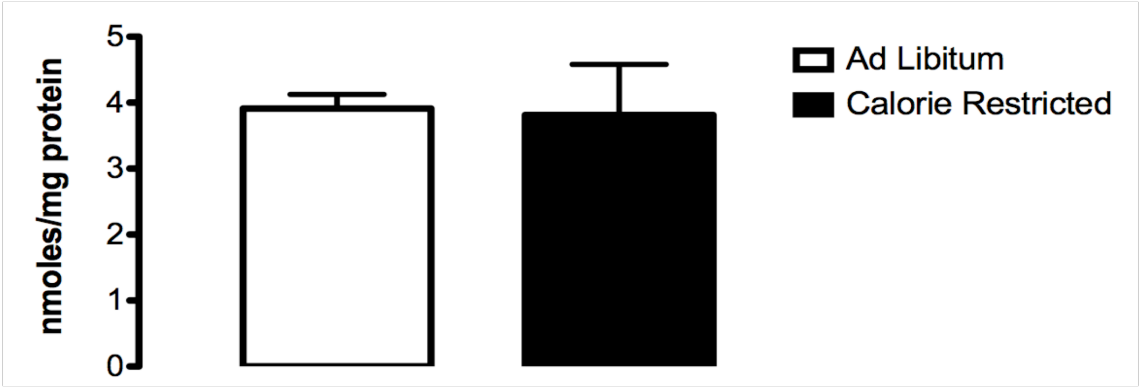


Fig. 32 Effect of caloric restriction on renal cortical homogenate malondialdehyde (MDA) levels: MDA levels were measured as thiobarbituric acid reactive substances (TBARS) in renal cortical homogenates by a colorimetric assay. Data is expressed as Mean±SEM of n=10-11. **p<0.05 vs. ad-libitum was considered significant* using *unpaired t-test.*

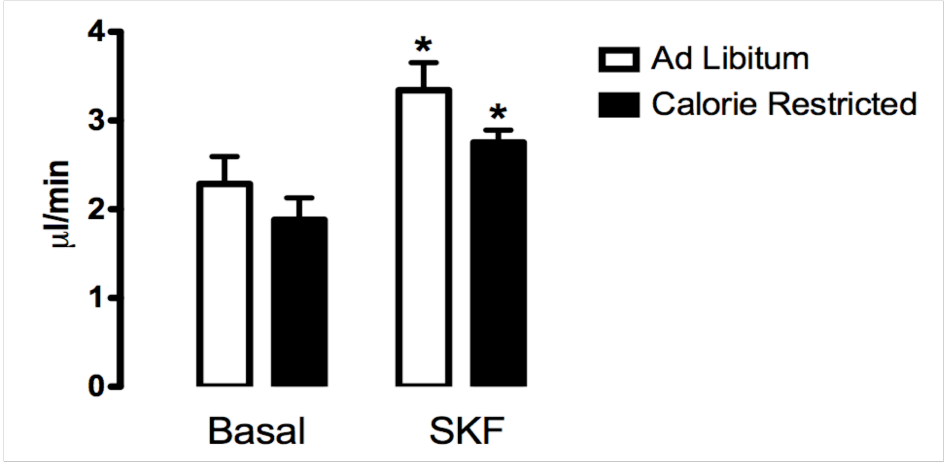


Fig. 33 Effect of caloric restriction on urine flow: Bars represent urine flow (µl/min) calculated using an average of two 30 min collections in basal phase and one 30 min collection during SKF38393 phase. Data is expressed as Mean±SEM of n=7. **p<0.05 vs basal was considered significant* using ANOVA followed by Newman-Keuls multiple test.

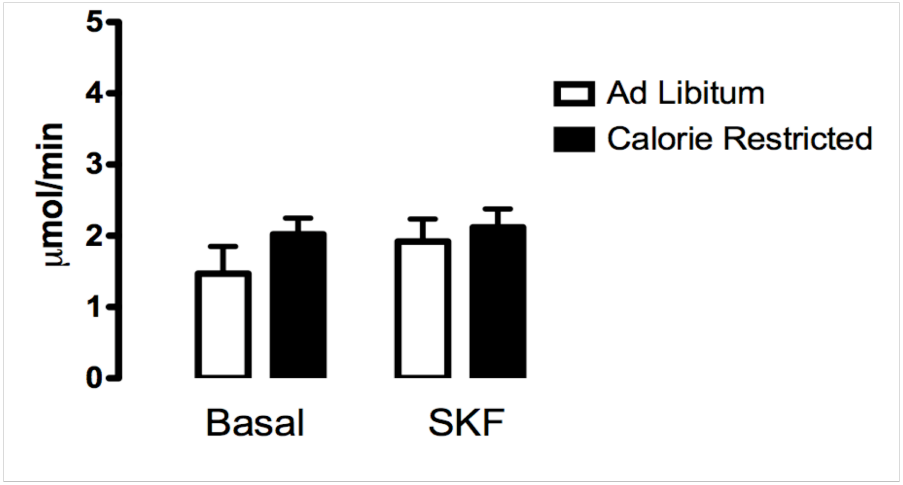


Fig. 34 Effect of caloric restriction on urinary sodium excretion ($U_{Na}V$): Bars represent $U_{Na}V$ (µmol/min) calculated using an average of two 30 min collections in basal phase and one 30 min collection during SKF38393 phase. Data is expressed as Mean±SEM of n=7.

5. DISCUSSION:

Our results show that, in obese Zucker rats, the exercise protocol employed in this study reduces oxidative stress by augmenting antioxidant defenses and also reduces albuminuria. However, it fails to lower blood glucose levels or improve insulin sensitivity, does not restore renal dopamine D1 receptor coupling to G proteins or renal dopamine D1 receptor mediated natriuresis and does not prevent the development of hypertension. This is despite the observation that the reduction in oxidative stress levels was enough to prevent the nuclear translocation of redox sensitive transcription factor NFκB that has been implicated in the impairment of natriuretic response to D1 receptor agonists in several models of hypertension, diabetes and aging (27, 30, 63, 64). This study provides evidence that reducing oxidative stress per se is not enough to restore renal D1 receptor function in this model. In addition to lowering oxidative stress, reducing insulin and/or blood glucose levels may play an important role in restoring renal D1 receptor function. Further, this study provides additional evidence that restoring D1 receptor function contributes to reducing blood pressure in obesity-associated hypertension.

The exercise protocol employed in our study did not reduce food intake or the gain in weight in obese rats. While some studies have reported moderate reductions in weight gain in female obese rats with swim training (217, 218),

several other groups have reported results that are similar to our study (133, 149, 194). Nevertheless, even in studies that reported reductions in weight gain, exercise only delayed but did not prevent the development of obesity (217). Inability of exercise to reduce weight gain, in this model, might be related to lack of attenuation of leptin resistance and the associated hyperphagia and impaired thermogenesis (16, 172). In a clinical trial involving children 8-14 years of age, Reinehr et al (186) have reported that plasma leptin concentrations are a predictor of overweight reduction with a lifestyle intervention. Although we did not measure leptin levels, others (117, 172, 221) have reported that exercise does not reduce leptin levels in these rats.

Exercise did not reduce blood pressure, fasting blood glucose or plasma insulin levels in obese rats. Exercise in both normal and overweight subjects has generally been associated with moderate decreases in blood pressure (220), improved insulin sensitivity and moderate reductions in blood glucose levels (166, 197). However, a meta-analysis study of exercise in type II diabetic patients showed that exercise failed to decrease blood pressure in this patient subset (206). In addition, even studies that report reductions in blood pressure, the size of this effect decreases with increasing body-mass-index (BMI) (220). A longer duration of exercise (14-22 weeks) has been shown to reduce systolic blood pressure in these rats using tail cuff method (17, 180). It is possible that the shorter duration of exercise (8 weeks) in our study precluded us from observing a

decrease in blood pressure. However, stress due to the restraining procedure involved in tail cuff method can limit the interpretation of data by providing false high values of blood pressure (213). Since exercised rats are more accustomed to handling and restraining in a narrow space compared to sedentary rats, it is possible that they displayed lesser stress during measurement of blood pressure. This might have been responsible for the apparent lower blood pressures in both the lean and obese exercised groups. Our results are similar to other studies in this model that did not notice any improvements in blood pressure with exercise (81, 221). Xiang et al (221) have reported a decrease in insulin levels with exercise. However, a number of other studies have reported no reductions in fasting plasma insulin levels with exercise in obese rats (38, 48, 53, 55, 117, 126, 169, 171, 203) as well as humans (206). While some studies have reported that insulin resistance in this model is refractory to exercise (149) others have reported improvements (133, 218). Further, even in studies reporting an improvement in insulin sensitivity, there were no reductions in fasting plasma insulin concentrations (53, 133, 169, 218). While our study involved 8 weeks of exercise at 10m/min, Xiang et al (221) exercised the rats for 4-5 weeks at 24m/min. However, the difference in intensity of exercise may not be responsible for the difference in the effect on insulin levels. Our results are in agreement with other authors who, using intensities ranging 20-26 m/min for more than 5 weeks, have reported no reductions in insulin levels with exercise (38, 53, 55, 117, 171).

Obese rats display higher HDL cholesterol levels and exercise paradoxically decreased HDL cholesterol levels in these rats. Higher HDL levels have normally been associated with lower LDL oxidation and better anti-inflammatory and anti-atherogenic profile (86). However, recent research suggests that HDL levels per se might not correlate with these beneficial effects (5, 44). Conditions like hyperglycemia, oxidative stress and chronic acute phase response seen in obesity and diabetes (both type I and II), can alter HDL composition and structure and actually promote LDL oxidation and inflammation (104, 161-164, 175-177, 212). In addition, our results are similar to those of Roberts et al (187), who reported that aerobic exercise in humans exhibiting metabolic syndrome actually decreases HDL levels while improving its anti-inflammatory properties. While exercise significantly reduced triglyceride levels in obese rats, it failed to decrease blood cholesterol levels in our study. A similar lack of effect of exercise on blood cholesterol levels has been reported in type II diabetic patients (206) and obese rats (117). Boor et al (38) did not observe any effect on triglyceride levels with 10 weeks of exercise in obese Zucker rats. The reason for differences between our study and the above study are not clear. However, we suspect that this might be due to the overnight fasting protocol employed in our study. Boor et al (38) do not mention if the rats were fasted overnight and the triglyceride levels in their study might reflect postprandial/random levels. In addition, our results are

similar to those of Frisbee et al (81) who, using 10 weeks of exercise and an overnight fasting period, observed a similar decrease in triglyceride levels.

Previously we have reported that anti-oxidant treatment of Fisher344 rats (64), obese rats (32, 144) and pro-oxidant treated SD rats (30) reduced oxidative stress and restored dopamine D1 receptor function. Further, exercise has been reported to augment physiological antioxidant mechanisms and improve renal dopamine D1 receptor function in old Fisher344 rats (19, 84). A critical role of NFκB nuclear translocation in impairment of dopamine D1 receptor function and its restoration following anti-oxidant treatment has been demonstrated in primary cultures (63) and pro-oxidant treated SD rats (30). In the present study, exercise reduced oxidative stress in obese rats as evidenced by increase in SOD activity in both plasma and proximal tubular homogenates and reductions in levels of the oxidative stress markers, urinary 8-isoprostane and renal malondialdehyde. These reductions in oxidative stress were enough to prevent the nuclear translocation of redox sensitive transcription factor NFκB that has been implicated in the development of renal dopamine D1 receptor dysfunction. However, we did not observe an improvement in dopamine D1 receptor mediated [^{35}S]GTPγS binding and sodium excretion, or reduction of blood pressure in this study.

The above results and also studies in other models suggest that oxidative stress might not be responsible for impairment of dopamine D1 receptor function and development of hypertension in all cases. For example, Escano et al (62) have shown that C57Bl/6J mice exhibit higher blood pressure compared to SJL/J mice, exhibit dopamine D1 receptor dysfunction and develop salt sensitive hypertension while SJL/J mice do not. However, when on high salt diet, SJL/J mice exhibit higher levels of oxidative stress compared to C57Bl/6J mice suggesting that oxidative stress is not always responsible for impairment of renal dopamine D1 receptor function and development of hypertension (62). In the light of these findings, it is possible that oxidative stress is not critical in the development of dopamine D1 receptor dysfunction and hypertension in obese Zucker rats.

Exercise reduced urinary albumin excretion in our study. In contrast, Boor et al (38) reported an increase in urinary protein levels with exercise. In this particular study the rats were placed in metabolic cages right after the last exercise session to collect urine used for measuring protein. Boor et al (38) acknowledge that this is a drawback of their study design, since it does not eliminate the acute effects of exercise. In addition, the authors report a decrease in number of glomeruli exhibiting protein droplets in their histopathological studies and this might actually indicate an improvement in terms of reducing proteinuria. In our study, we collected urine from the bladder 48hrs after the last bout of exercise to

eliminate the acute effect of exercise on proteinuria. Further, as pointed out by Boor et al, a number of other studies support our observation that exercise reduces proteinuria (19, 51, 219).

In our study, exercise reduced proximal tubular oxidative stress, reduced albuminuria and decreased proximal tubular NFκB activation in obese rats. While albuminuria is used as a marker of renal (glomerular) injury, it is reported that albuminuria can by itself initiate a vicious cycle of proximal tubular injury and oxidative stress (116). Morigi et al (152) have shown that albumin induces proximal tubular injury via a mechanism involving generation of reactive oxygen species (ROS) and activation of NFκB. Also, increased oxidative stress in SJL/J mice makes them susceptible to increased albuminuria and renal injury when compared to C57Bl/6J mice (62). In addition, Quigley et al (182) have reported that renal injury in obese (db/db) mice might result from increased oxidative stress rather than increased blood pressure. Our findings are similar to the above reports in that a reduction in oxidative stress with exercise was associated with decrease in albuminuria but not blood pressure.

Therefore, this study offers evidence that exercise is beneficial in reducing oxidative stress, nuclear translocation of NFκB and renal injury in obese Zucker rats. However, the reductions in oxidative stress are not enough to prevent renal dopamine D1 receptor dysfunction and development of hypertension. In addition,

considering previous studies (25, 26, 29), we predicted that reducing insulin levels may be an important pre-requisite to prevent renal dopamine D1 receptor dysfunction that contributes to development of hypertension in this model.

Another piece of evidence indicating the primary role of insulin was our study in $Lepr^{fa/fa}$ **(**obese) Zucker rats, at three to four weeks of age. At this age, the $Lepr^{fa/fa}$ **(**obese) Zucker rats are hyperinsulinemic and dyslipidemic but do not display hyperglycemia or significantly elevated blood pressure. At this age, proximal tubular SOD activity is decreased when compared to $Lepr^{-/-}$ (lean) Zucker rats. However, other antioxidant components like reduced-glutathione (GSH) and hemeoxygenase-1 (HO-1) were not decreased when compared to lean rats. In addition, several markers of oxidative stress like plasma and urinary 8-isoprostane, proximal tubular protein carbonylation, proximal tubular protein nitrosylation, and the ratio of reduced-glutathione to oxidized-glutathione were similar when compared with lean rats. In addition, the nuclear translocation of redox sensitive transcription factor NFκB was similar in both the groups. Therefore, $Lepr^{fa/fa}$ **(**obese) Zucker rats, at three to four weeks of age, do not display oxidative stress or increased nuclear translocation of NFκB. Furthermore, three-week-old obese Zucker rats do not display downregulation of dopamine D1 receptor compared to age matched lean controls. Despite this, dopamine D1 receptor mediated inhibition of Na^+/K^+ ATPase at this age is impaired when

compared to lean rats. Defective dopamine D1 receptor-G protein coupling, evidenced by inability of dopamine D1 agonist (SKF38393) to stimulate [^{35}S]GTPγS binding, might be responsible. Considering these results in three-week-old obese Zucker rats, the results from our exercise study, and earlier studies using antioxidants (21, 32), we concluded that reducing hyperinsulinemia might be a prerequisite to restore dopamine D1 receptor function.

Another lifestyle intervention that is recommended for reducing weight gain and lowering blood pressure is restriction of caloric intake (155, 160). In addition, adequate amount of caloric restriction (~50% restriction) in obese Zucker rats, starting at an early age, is associated with decrease in insulin levels (22, 141). Therefore, we hypothesized that caloric restriction in obese Zucker rats from an early age will reduce insulin levels, restore renal dopamine D1 receptor function and reduce blood pressure.

Caloric restriction in obese Zucker rats, starting from seven weeks of age, decreased the gain in weight in these rats when compared with ad-libitum group. This reduction in the rate of weight gain was associated with a reduction in triglyceride levels and a marginal decrease in ratio of fat to body weight. In addition, caloric restriction decreased fasting insulin levels and improved insulin sensitivity. However, these changes were not associated with restoration of natriuresis in response to dopamine D1 receptor agonist (SKF38393) or a

decrease in blood pressure. Caloric restriction reduced insulin levels by ~50% when compared with ad libitum group. Our observations regarding insulin levels are similar to studies from other labs (22, 141) and offer further evidence that caloric restriction to lean levels, starting at an early age, reduces insulin levels and improves insulin sensitivity. However, our observations regarding blood pressure are in contrast to those of Maddox et al (141) who observed a reduction in blood pressure in conscious rats using the tail cuff method. We measured blood pressure via a carotid artery catheter in anesthetized rats. Our results are similar to those of Kurtz et al (131) who also measured arterial pressure directly via femoral catheters in conscious rats, thus ruling out the possibility of interference from anesthesia. Another explanation for the differences between our study and that of Maddox et al (141) might be the drawbacks presented by the tail cuff method (213). The reductions in blood pressure reported by Maddox et al (141) might potentially reflect the variable reactivity of blood pressure to stress rather than treatment (caloric restriction) effect (213).

While caloric restriction reduced fasting insulin levels and improved insulin sensitivity it failed to reduce oxidative stress. Caloric restriction failed to increase SOD activity in both the plasma and renal cortical homogenates. Caloric restriction failed to decrease protein carbonylation, protein nitrosylation, and malondialdehyde levels in renal cortical homogenates, when compared with ad-libitum group. Although caloric restriction marginally decreased (by ~20%)

plasma 8-isoprostane levels, it increased urinary 8-isoprostane levels approximately three fold. These results might indicate a difference in the effect of caloric restriction on systemic oxidative stress versus local effect in the kidney. In fact, caloric restriction might actually increase renal oxidative stress as is evidenced by increased urinary 8-isoprostane levels. An alternative explanation for the increase in urinary 8-isoprostane levels might be the fact that caloric restriction has been reported to decrease the protein levels of cyclo-oxygenase 1 and 2 (COX 1/2) (183). This decrease in COX 1/2 might increase the arachidonic acid pool available for oxidative conversion to 8-isoprostane. Irrespective of the source of the increased urinary 8-isoprostane levels, this increase might have important physiological and possibly pathophysiological implications. Isoprostanes are potent vasoconstrictors that can decrease renal blood flow and glomerular filtration rate (GFR) (23, 153, 154). This vasoconstrictor effect, at low nanomolar concentration, seems to be renal specific and not systemic because an acute infusion that achieves this concentration does not elevate blood pressure (153). The effect of isoprostanes on electrolyte reabsorption, specifically dopamine-mediated inhibition of sodium reabsorption, is still unexplored. Regardless, these results indicate that caloric restriction failed to decrease oxidative stress and did not restore dopamine D1 receptor mediated natriuresis in obese Zucker rats.

Therefore, caloric restriction in obese Zucker rats decreases insulin levels and improves insulin sensitivity. However, caloric restriction does not reduce oxidative stress, does not restore renal dopamine D1 receptor function, and does not reduce blood pressure in these rats.

LIMITATIONS:

The above discussion needs to be qualified by the complexity presented by the obese Zucker rat as a model of metabolic syndrome. Although our study and previous studies indicate a critical role of hyperinsulinemia and oxidative stress in the impairment of renal dopamine D1 receptor function in this model, it is possible that other factors play an important role. In addition, it is possible that restoration of renal dopamine D1 receptor function by tempol is related to the correction of condition(s) other than hyperinsulinemia and oxidative stress. It follows that both exercise and caloric restriction might have failed to restore renal dopamine D1 receptor function as a result of the inability to correct the above condition(s) that conceivably was/were corrected by tempol.

6. SUMMARY AND CONCLUSIONS:

6.1 SUMMARY:

In obese Zucker rats

1. Hyperinsulinemia, dyslipidemia and impairment of renal dopamine D1 receptor function precede the development of hyperglycemia, oxidative stress and the associated increase in nuclear translocation of NFκB, and hypertension.

2. Exercise is beneficial in improving lipid profile, reducing oxidative stress, reducing nuclear translocation of NFκB and reducing renal injury. However, exercise does not reduce rate of weight gain, does not decrease insulin levels or improve insulin sensitivity, does not correct renal dopamine D1 receptor dysfunction and does not reduce blood pressure.

3. Caloric restriction improves lipid profile, reduces the rate of weight gain, reduces insulin levels and improves insulin sensitivity. However, caloric restriction does not decrease oxidative stress, does not correct renal dopamine D1 receptor dysfunction and does not reduce blood pressure.

6.2 CONCLUSION:

Considering the above studies and previous literature, we conclude that both hyperinsulinemia and oxidative stress can independently impair renal dopamine D1 receptor function contributing to the development of hypertension in obese Zucker rats. As a corollary, simultaneously reducing both oxidative stress and hyperinsulinemia might be required to restore renal dopamine D1 receptor function and attenuate hypertension. Either exercise or caloric restriction, independently, fails to simultaneously correct both hyperinsulinemia and oxidative stress in these rats. Therefore, renal dopamine D1 receptor impairment and development of hypertension cannot be corrected/attenuated by either intervention in isolation. A combination of both exercise and caloric restriction may be required to restore renal dopamine D1 receptor function and lower blood pressure in obese Zucker rats.

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